

Controlled Conversion of an Immortalized Mesodermal Progenitor Cell Towards Osteogenic, Chondrogenic, or Adipogenic Pathways

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Abstract. The teratocarcinoma-derived C1 clone behaves as a mesodermal tripotential progenitor cell whose choice of fate, either osteoblast, chondroblast, or adipoblast, is strictly dependent on the spatial organization of the cells and the nature of the induction. In the absence of cell contact before the addition of inducers, the C1 cells maintain a stable undifferentiated phenotype while expressing potential regulators of embryonic mesodermal stem cell fate such as M-twist and Id₁. Upon establishment of cell contacts before the induction of differentiation, the early genes characteristic of the three fates become expressed. In the presence of β glycerophosphate and ascorbate, provided the cells have formed aggregates, 95% of the C1 cells mineralize with a kinetics of gene expression close to that of osteoblasts (Poliard, A., D. Lamblin, P. J. Marie, M. H. Buc, and O.

Kellermann. 1993. *J. Cell Sci.* 106:503–512). With 10^{-6} M dexamethasone, 80% of the same aggregates differentiate into foci of chondroblast-like cells. The kinetics of expression of the genes encoding type II, IX, X, and XI collagens, aggrecan and link protein during the conversion towards cartilage hypertrophy resembles that accompanying in vivo chondrogenesis. The synergistic action of dexamethasone and insulin convert most confluent C1 cells into functional adipocytes and induce a pattern of gene expression close to that reported for adipoblast cell lines. The C1 clone with its capacity to differentiate along three alternative pathways with high frequency, therefore appears as a valid in vitro model for deciphering the molecular basis of mesoblast ontogeny.

THE molecular and cellular mechanisms underlying the sequential differentiation of mesodermal cells have been the object of numerous studies. For this purpose, cell culture models containing presumptive progenitors capable of giving rise to different mesodermal cell types have been established from embryonic tissues (Konieczny and Emerson, 1984; Grigoriadis et al., 1988; Taylor and Jones, 1979), bone or muscle primary cultures (Yamaguchi and Kahn, 1991; Jakob et al., 1978), mouse teratocarcinoma (Darmon et al., 1984; Atsumi et al., 1990), or bone marrow stroma (Bennett et al., 1991; Beresford, 1989; Fridenstein, 1976). In such cultures, subsets of the same cell line often concurrently express several phenotypes and may spontaneously favor a single phenotype. Such behavior has allowed one to follow the development of myogenic, chondrogenic, adipogenic, or osteogenic colony-forming cells. However, the mesodermal progenitor cell itself, which presumably gives rise to all of these lineages, has not yet been identified.

The clonal frequency of conversion of the pluripotential

cells towards one phenotype always appears less than 40%, even under culture conditions favoring one restricted lineage (Taylor and Jones, 1979; Darmon et al., 1984; Grigoriadis et al., 1988; Bennett et al., 1991). For example, the mouse embryonic cell line C3H10T1/2, which has proven to be a powerful in vitro model for isolation of myogenic determination factors does not escape this behavior. In this cell line, 5-azacytidine treatment mediates a stable, inheritable change through an irreversible DNA hypomethylation which activates one of the three regulatory programs leading to myofibers, chondrocytes, or adipocytes. Incorporation of 5-azacytidine into the DNA molecule is sufficient to convert 30–40% of the parental 10T1/2 cells; 25% becoming myofibers, 7% adipocytes, and 1% chondrocytes-like cells (Taylor and Jones, 1979; Konieczny and Emerson, 1984). It has been proposed that the parental 10T1/2 cells behave as pluripotential cells blocked in the mesodermal developmental pathway. Such a block is overcome by a direct action on DNA, mediated by independent demethylation events. This action is sufficient to directly convert the stem cell into three progenitors each determined towards one of the three lineages. Recently, bone morphogenetic protein-2, one of the signaling molecules taking part into the initiation of embryonic bone

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and cartilage formation (Reddi, 1994), has been shown to be also capable to induce the codifferentiation of the C3H10T1/2 cells into fat, cartilage, and bone (Wang et al., 1993b; Ahrens et al., 1993). But the frequency of differentiation towards one particular phenotype remains low particularly in the case of cartilage. Therefore mutually exclusive and homogenous differentiation pathways in response to changing signals have never been obtained for a clonal mesodermal progenitor cell.

One method of choice to unravel some of the complexity of cell type determination and to evaluate the nature of external influences capable of triggering the alternative decisions of a mesodermal stem cell, would be to isolate an immortalized cell line which is stable in a stem cell state and capable of further homogeneous differentiation after *in vitro* appropriate induction. We previously reported a successful strategy for immortalizing precursor cells from an embryonal carcinoma cell line (EC)¹ with the help of a recombinant plasmid PK₄ containing the early genes of SV40 under the control of the adenovirus E1A promoter (Kellermann and Kelly, 1986; Kellermann et al., 1987). This promoter allows the expression of SV40 oncogenes in early embryonic cells at the very time differentiation of multipotential EC cells is induced and, consequently, promotes the immortalization of committed precursors along neuroectodermal, endodermal, and mesodermal pathways (Kellermann et al., 1987; Buc-Caron et al., 1990; Kellermann et al., 1990). Our idea was that such an immortalization step could freeze a progenitor cell in a particular determination state, thereby providing a source of homogeneous clonal stem cells capable of further differentiating towards different fates upon induction by appropriate stimuli.

Among the EC cell lines that we could transform, one of them, 1003-PK₄, enabled us to select the C1 clone. The method of cloning of the C1 cell by limiting dilution from an island of cells resembling the somitic embryonic mesoderm has been described (Kellermann et al., 1990). This island was mainly composed of mesoblastic cells able to differentiate into either myogenic, fibrogenic, adipogenic, or osteogenic derivatives. The mesodermal clone C1 never spontaneously differentiates *in vitro*. The osteogenic potential of C1 was first established *in vivo* by the production of osteosarcoma. *In vitro*, when cultured in three dimensional (3D) aggregates, C1 cells were shown to engage in a synchronous manner through the well-characterized steps of osteogenic differentiation upon addition of both ascorbic acid (AA) and β -glycerophosphate (β GP). Remarkably, under such conditions, more than 95% of the aggregates secrete a type I Col extracellular matrix which progressively mineralizes. The kinetics of osteogenic differentiation has already been well characterized at the level of both the histology of the mineralizing aggregates and the expression of a set of genes representative of bone matrix proteins (Kellermann et al., 1990; Chentoufi et al., 1993; Poliard et al., 1993). Under AA and β GP culture conditions, C1 cells appear univocally committed towards

1. *Abbreviations used in this paper:* 3D, three dimensional; AA, ascorbic acid; A2COL6, α 2 chain of type VI Collagen; ALBP, adipocyte lipid-binding protein; β GP, β -glycerophosphate; Col, collagen; DEX, dexamethasone; EC, embryonal carcinoma cells; GAPDH, glyceraldehyde phosphate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; LPL, lipoprotein lipase.

the osteogenic pathway, the lack of a type II collagen matrix detection further indicating the total absence of cartilage formation in the aggregates.

In the present study, we show that the C1 cells can also follow two other pathways of differentiation, giving rise under the action of the appropriate inducers to either chondroblast-like or adipocyte-like cells. The high frequency of differentiation of C1 precursor cells allow us to follow the gene expression pattern accompanying the transition towards the terminal phases of chondrogenic or adipogenic differentiation.

In conclusion, the C1 clone has the properties of a tripotential mesodermal progenitor cell having the capacity of both multiply in a stable manner and produce a progeny whose choice of fate is driven by the nature of extracellular signals.

Materials and Methods

Cell Culture and Differentiation

C1 cells were cultured as described (Kellermann et al., 1990). To form 3D aggregates, C1 cells were seeded at 3×10^5 cells per 10 ml of DME supplemented with 10% FCS on untreated plastic dishes. After 9 d (day 0), the 3D clusters (4,000–5,000 per dish) were refed with DME supplemented with 1% FCS and induced to differentiate (a) along the osteogenic pathway by the addition of 50 μ g/ml AA and 7 mM β GP, or (b) along the chondrogenic pathway by the addition of 10^{-6} M dexamethasone (DEX). To induce adipocyte differentiation, C1 cells were grown to confluence in DME supplemented with 10% FCS and further treated by 10^{-7} M DEX and 10^{-6} M insulin in DME supplemented with 1% FCS. Cells were refed every 3 d with or without pharmacological effectors. Exponential cultures were established by dissociating confluent cells, and seeding them at low density (5×10^2 cells per cm²) without cell–cell contacts during 3 d.

DNA Probes

The following DNA probes were used: mouse type I and type II collagen (Col) cDNA EcoRI–HindIII inserts of 321 and 405 bp, respectively (Metsäranta et al., 1991); mouse type X Col cDNA HindIII insert of 360 bp (Apte et al., 1992); rat aggrecan cDNA PstI insert of 710 bp (Doege et al., 1987); rat link protein cDNA EcoRI insert of 820 bp (Doege et al., 1986; Rhodes et al., 1988); mouse type XI Col cDNA XbaI–XhoI insert of 800 bp (Stubbs et al., 1993); rat osteocalcin (OC) cDNA EcoRI insert of 520 bp (Yoon et al., 1988); *Xenopus borealis* histone H4 genomic HindIII–XbaI fragment of 594 bp; rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA PstI insert of 1.3 kb (Fort et al., 1985); a probe for type IX Col corresponding to a cDNA EcoRI insert of 1 kb; mouse α 2-chain of type VI Col (A2COL6) cDNA EcoRI insert of 2.9 kb (Dani et al., 1989); mouse lipoprotein lipase (LPL) cDNA EcoRI insert of 1.4 kb (Kirchgesner et al., 1987); mouse adipocyte lipid-binding protein (ALBP) cDNA pSTI insert of 600 bp (Spiegelman et al., 1983); mouse glycerol-3-phosphate dehydrogenase (GPDH) HindIII gene fragment of 0.8 kb (Ireland et al., 1986); mouse Twist cDNA XbaI–EcoRI insert of 420 bp (Wolf et al., 1991); and Id cDNA SmaI insert of 920 bp (Benezra et al., 1990).

RNA Isolation and Northern Blot Analyses

Total RNA was isolated and Northern blot analyses were performed as described (Poliard et al., 1993). Poly A⁺ RNA was prepared with the mRNA Purification Kit (Pharmacia Biotechnology, Piscataway, NJ).

Histochemical, In Situ, Immunocytochemical, and Electron Microscopy Studies

For histochemical analyses, the formed nodules were fixed in 90% ethanol at 4°C and embedded in glycolmethacrylate. 5- μ m sections were stained with toluidine blue. Immunocytochemical analyses and *in situ* hybridization studies of C1 cell aggregates were performed as described (Poliard et al., 1993). Aggregate sections were treated for 1 h with 0.3 U/ml of chondroitinase ABC (Boehringer Mannheim Corp., Indianapolis, IN) in 0.1 M

Tris acetate buffer (pH 7.3). A permeabilization step with cold ethanol 95% was then performed. Indirect immunofluorescence was carried out using as primary antibodies (a) a rabbit anti-mouse type I Col polyclonal antiserum (Chemicon International Inc., Temecula, CA); (b) a monoclonal anti-chicken type II Col (Mayne et al., 1994); (c) a specific monoclonal antibody against cartilage proteoglycan (Miles, Bayer Diagnostics); (d) a goat anti-mouse osteocalcin antibody (BTI, Stoughton, MA). Specific secondary antisera coupled to fluorescein were used to visualize the sites of primary antibody binding. Electron microscopy was performed as described (Kellermann et al., 1990).

Lipolysis Assays

Lipolysis experiments were carried out as described (Forest et al., 1983). Cells were induced to differentiate in 35-mm tissue culture dishes. After 17 d, lipid labeling was performed during 48 h by adding 0.5 μ Ci 1- 14 C]acetate (ICN, 40–60 mCi/mmol) in 2.5 ml DME supplemented by 1% FCS, 10^{-7} M DEX, and 10^{-6} M insulin. The labeling procedure was repeated once. For lipolysis assays, cells were rinsed twice at 37°C with PBS, and then incubated in 2 ml DEM containing 1% bovine serum albumin (Sigma; fatty acid-free). Radioactivity present in the medium was measured 30 min later (time 0) and the effectors were added. Samples of the

medium were checked for the presence of fatty acid-linked radioactivity 1 and 2 h later.

Results

Specific Phenotypic Conversion of C1 Mesodermal Cells into Osteocyte-, Chondrocyte-, or Adipocyte-like Cells Depends on Distinct Epigenetic Conditions

In vivo embryonic skeleton formation depends critically on the condensation of mesodermal stem cells. Accordingly, C1 cells were cultured in 3D aggregates to search for the induction of either osteogenic or chondrogenic in vitro differentiation. As already reported, when cultured as nodules in the presence of AA and β GP, C1 cells deposit a type I collagenous extracellular matrix which progressively mineralizes (Fig. 1 B) and forms hydroxyapatite crystals. After 2 d of treatment, the progressive develop-

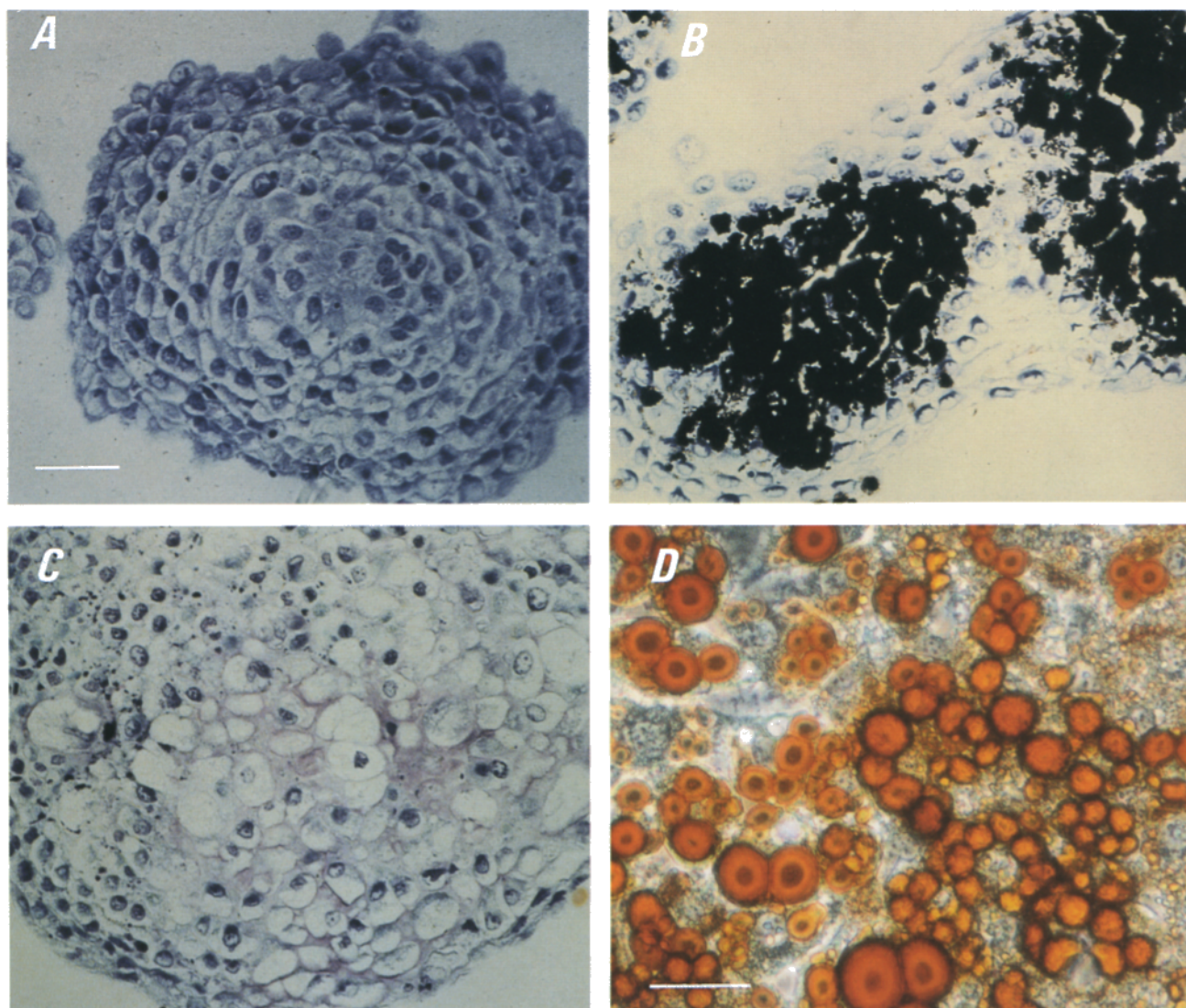


Figure 1. Phenotypic conversion of C1 cells into osteocyte-, chondrocyte-, and adipocyte-like cells. Toluidine blue staining on nodule sections (A) control at 50 d of culture; (B) β -GP and AA-treated nodules at 20 d of culture showing matrix mineralization stained by Von Kossa; (C) DEX-treated nodules at 50 d of culture showing chondrocytes contained in lacuna separated by an extracellular matrix; (D) Fat droplets stained with oil red O: monolayer culture of C1 cells after 20 d of treatment with DEX and insulin. Bars: (A, B, and C) 10 μ m; (D) 50 μ m.

ment of mineralizing foci occurs in a partially synchronous manner in almost 100% of the C1 aggregates (Kellermann et al., 1990; Chentoufi et al., 1993; Poliard et al., 1993).

In the presence of DEX (10^{-6} M), after a minimum of 25 d, C1 aggregates show foci of chondroblast-like cells with a cuboidal morphology and a typical metachromatic and perilacunar staining with toluidine blue (Fig. 1 C). More than 80% of the C1 aggregates acquires foci of chondroblast-like cells heterogeneous in size. As in vivo maturing chondrocytes, cells reside in lacunae surrounded by an extracellular matrix of thin type II Col fibrils and electron-dense small condensations of putative proteoglycans (Fig. 2). After 3 mo of exposure to DEX, the lacunae progressively become larger and the chondrocytes undergo terminal differentiation to produce hypertrophic cells (not shown).

When monolayers of confluent C1 cells are exposed to both DEX (10^{-7} M) and insulin (10^{-6} M), two known positive regulators of adipogenic differentiation (Kodama et al., 1982; Ringold et al., 1986; Ailhaud et al., 1992), the adipocyte differentiation occurs in a reproducible and almost synchronous manner. After 17 d, more than 80% of the C1

cells are positively stained by oil Red O which specifically reveals the presence of triglycerides (Fig. 1 D).

Noteworthy, in the absence of inducers, the clonal C1 cells maintain a stable phenotype for months (Fig. 1 A). Matrix formation, Ca^{++} accumulation and mineralization are never observed within untreated C1 nodules (Chentoufi et al., 1993). After 120 d of culture less than 5% of the control aggregates initiate chondrogenic differentiation by secreting a weak type II Col extracellular matrix deprived of proteoglycan (not shown). Finally, in long term monolayer cultures (over 3 mo) C1 cells never spontaneously differentiate into fat-containing cells.

These observations suggest that the clone C1 behaves as a tripotential mesodermal precursor whose choice of fate depends on both the spatial organization of the cells and the nature of the induction. To further strengthen this conclusion, the patterns of gene expression underlying these alternative phenotypic conversions were examined.

Chondrogenic Differentiation

Cartilage Specific Gene Expression Occurs during the Dif-

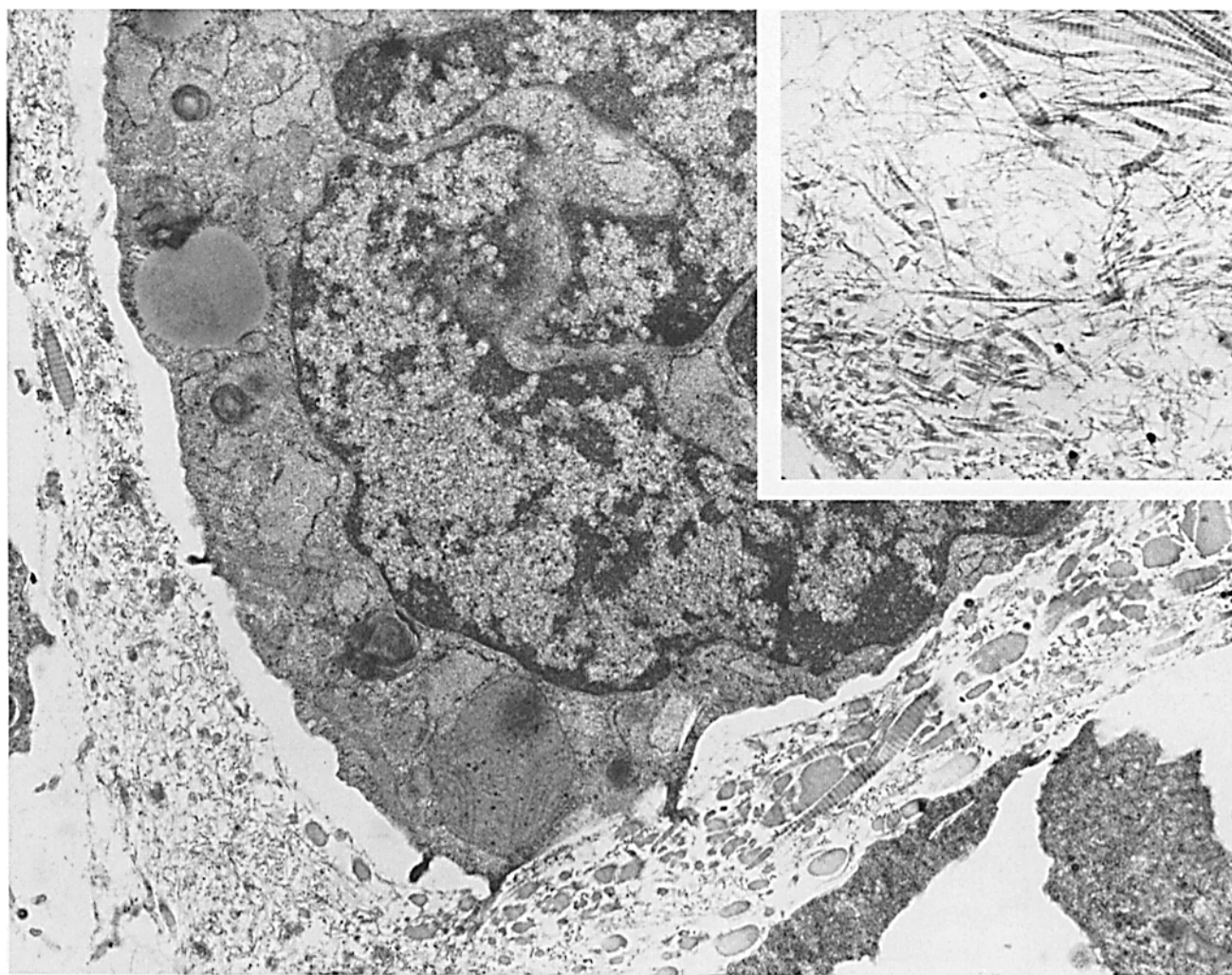


Figure 2. Electron micrograph of C1 chondrocyte performed on a nodule section after 60 d of DEX treatment. The cell resides in a lacuna, cellular processes appear along the chondrocyte membrane. (*inset*) The extracellular matrix consist of a network of thin type II collagen fibrils, banded fibrils, and small electron dense condensations of proteoglycans. Bar, 1 μm .

ferentiation of Committed C1 Cells Towards Mature Chondrocytes. Steady-state levels of transcripts encoding type I, II, IX, X, or XI Col, large proteoglycan core protein and link protein were evaluated by Northern blot analysis, during C1 chondrogenic differentiation.

At day 0, type I, II, XI Col mRNAs are already detectable, independent of the presence or absence of DEX (Fig. 3). Upon addition of 10^{-6} M DEX, the steady-state levels of these transcripts considerably change over the following 85-d period (Fig. 3). In untreated aggregates, these mRNAs do not significantly vary (not shown).

Type I Col mRNA, the major constituent of bone matrix, is known to be expressed in chondroprogenitors cells (Kosher et al., 1986; Tacchetti et al., 1987). Around day 25, a time which coincides with the development of cartilage foci, type I Col mRNA level declines. At day 50 the amount of type I Col mRNA has dramatically decreased

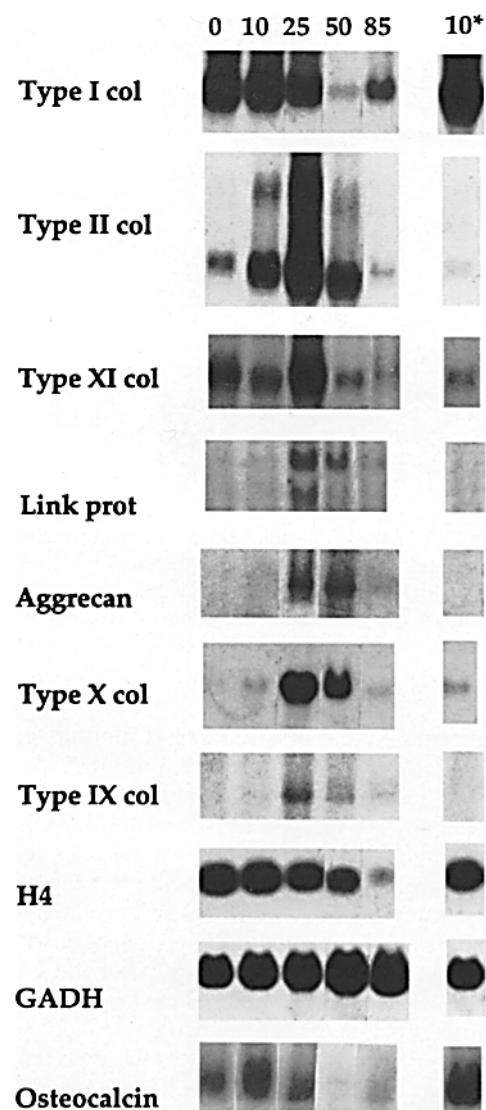


Figure 3. Kinetics of gene expression during chondrogenic differentiation analyzed by Northern blotting: type I, II, XI, X, and IX Col; Aggrecan; Link protein; Osteocalcin; histone H4; GAPDH. Total RNA were extracted from 3D aggregates after 0, 10, 25, 50, and 85 d of DEX treatment or after 10 d of treatment with DEX and retinoic acid (10*).

(Fig. 3). At day 85 however it increases again. This coincides with the maturation of the C1 cells to fully differentiated hypertrophic cells. The steady-state level of osteocalcin mRNAs parallels those of type I Col during the kinetics (Fig. 3).

The level of mRNA encoding type II Col, a major component of cartilage matrix, increases until day 25, before the first morphologic differentiation of the cells (Fig. 3). At later times, it decreases continuously until the end of the culture. In a similar way, the level of type XI Col transcripts, encoding one of the minor cartilagenous collagens, peaks on day 25 and decreases thereafter.

In contrast to type II and XI Col mRNAs, transcripts encoding other cartilage specific matrix proteins are not detected in the early stage of C1 differentiation before the onset of visible chondrogenesis. Expression of all monitored cartilage-specific genes is detected at day 25 (Fig. 3). Transcripts encoding type IX and also type X Col, a marker of the fully differentiated chondrocytes, are still expressed at day 50 and, at low level, at day 85.

A major structural component of the extracellular matrix of cartilage is aggrecan, a proteoglycan capable to form aggregates with hyaluronic acid (Doege et al., 1987). The interaction of aggrecan with hyaluronic acid is stabilized by the link protein. The mRNA encoding the proteoglycan core protein, as well as the two mRNAs of the link protein (Rhodes et al., 1988) are expressed mostly at day 25. These latter transcripts are still measurable at day 50 and become undetectable thereafter (Fig. 3). Therefore, in parallel to the progression of the C1 aggregates through chondrogenic differentiation, the steady-state levels of the mRNA encoding extracellular matrix components undergo large variation.

Interestingly, if retinoic acid (5×10^{-7} M) is added in combination with DEX to C1 nodules, chondrogenic differentiation does not occur. Type II Col mRNA rapidly declines and is almost undetectable as early as day 10 (Fig. 3). This is consistent with the observations that retinoic acid interferes with cartilage differentiation in vivo (Solursh, 1989) and in skeletal cells (Horton et al., 1987; Bernier and Goltzman, 1993).

Type I and II Collagen mRNAs Acquire Distinct Cellular Distributions before the Onset of Visible Chondrogenic Differentiation of C1 Cell. In vivo, the initial stages of chondrogenesis include a switch in Col synthesis, from type I to type II Col (Kosher et al., 1986; Tacchetti et al., 1987). To observe this switch at the single-cell level, an in situ hybridization analysis was performed.

At day 0, before the addition of DEX, type I and II Col transcripts are present in the majority of the cells, suggesting that C1 precursor cells coexpress the two types of collagen transcripts (as in Fig. 4 A). At day 17, before the onset of visible chondrogenesis, type I Col transcripts are still uniformly distributed over the aggregates in a way similar to that observed at day 0 (Fig. 4 A). Regarding type II Col, an increase in the silver grain density becomes visible over the condensed cells area (Fig. 4 B). These cells may correspond to the condensation of presumptive prechondrogenic cells. This observation is in agreement with the overall enhancement of type II Col mRNAs as measured by Northern blot (25 d) (Fig. 3). At day 50, the type I Col mRNA signal has become almost undetectable. The hy-

bridization signal for type II Col is still intense over the differentiated cartilage foci (not shown).

Immunochemical Localization of type I and II Collagens and of the Aggrecan Protein during Chondrogenic Differentiation of C1 Cells. To establish relationships between the time course of gene expression and that of cartilage matrix formation, we have followed the cellular localization of three important matrix proteins: type I and II Col and aggrecan.

As already pointed out for osteogenic differentiation, in exponentially growing C1 cells, none of the differentiation markers are expressed (Fig. 5). As soon as cells establish contacts, type I and II Col become detectable with specific antibodies (Poliard et al., 1993, and Fig. 6 A, respectively). The signal is weak but significant when compared to control experiments performed without primary antibody or with nonimmune serum. The synthesis of the two Col observed in aggregate sections (Fig. 6 A; Poliard et al., 1993) as well as in confluent cells (for type I Col; Poliard et al., 1993) does not require addition of any inducer. After the cells have established contacts and the two Col have become detectable within most of the cells, the type of extracellular matrix produced by the C1 cells critically depends on the nature of inducers.

Upon commitment by the addition of AA and β GP towards osteogenic differentiation, type I Col is assembled and forms an extracellular matrix as early as day 2. It is profusely secreted by day 5, whereas at the same time type II collagen is no longer detectable. The localization of os-

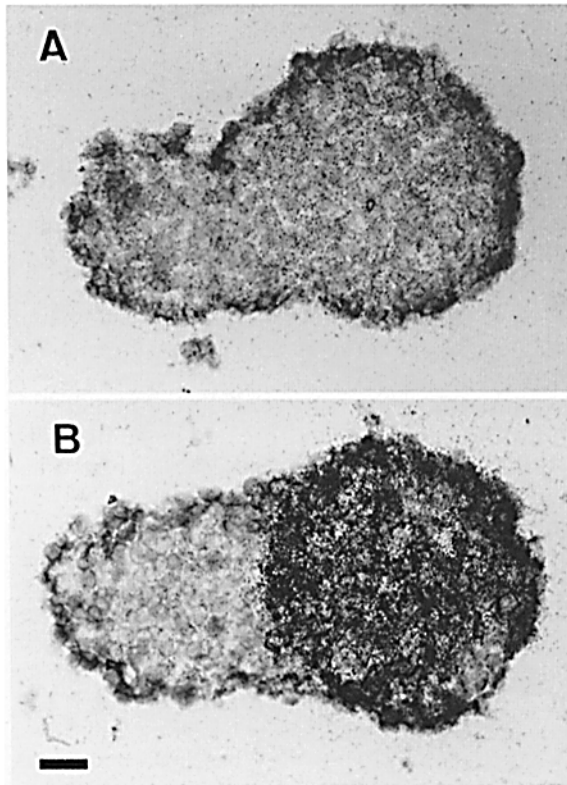


Figure 4. Autoradiographs of serial sections of aggregates treated by DEX for 17 d processed for in situ hybridization. (A) Type I Col transcripts appear uniformly distributed. (B) Type II Col transcripts appear overexpressed over the condensed cell area. Bar, 30 μ m.

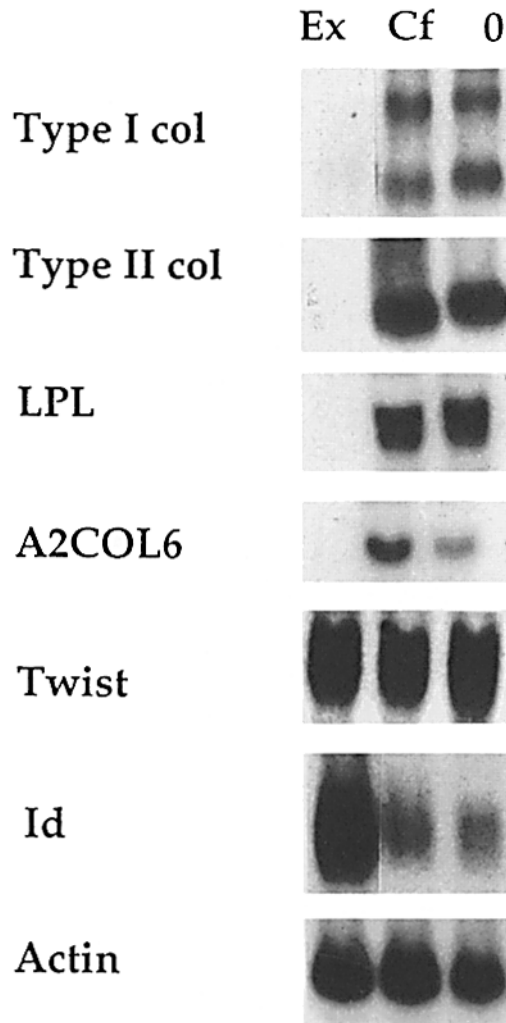


Figure 5. Northern blot analysis of gene expression in untreated C1 cells before or after establishment of cell contacts. Total RNA or Poly A⁺ RNA for Id and M-Twist were extracted from exponential (Ex), confluent (Cf), or untreated control aggregates (3D): Type I Col; Type II Col; LPL; A2COL6; M-Twist; Id and β -Actin.

teocalcin superimposes on that of type I Col. (Chentoufi et al., 1993; Poliard et al., 1993). Upon addition of DEX, co-expression of type I and II Col are maintained until day 25 (Fig. 6 B). Between day 30–40, type I Col vanishes in 90% of the nodules, whereas type II Col and proteoglycan get settled into an extracellular network (respectively, Fig. 6, C–D and 6, E–F). After day 50, type II Col and proteoglycan matrix systematically delineates cartilage foci of heterogeneous sizes recognizable in more than 80% of the C1 nodules. At all these times, osteocalcin is never detected by immunofluorescence analysis (not shown).

Interestingly, at day 85, while chondrocytes undergo terminal differentiation, type I Col is synthesized de novo, being accumulated in the extracellular space in almost 50% of the aggregates (not shown). This observation is in agreement with the significant reappearance of type I Col mRNAs (Fig. 3).

Adipocyte Differentiation

Changes in Gene Expression during Adipocyte Differentiation

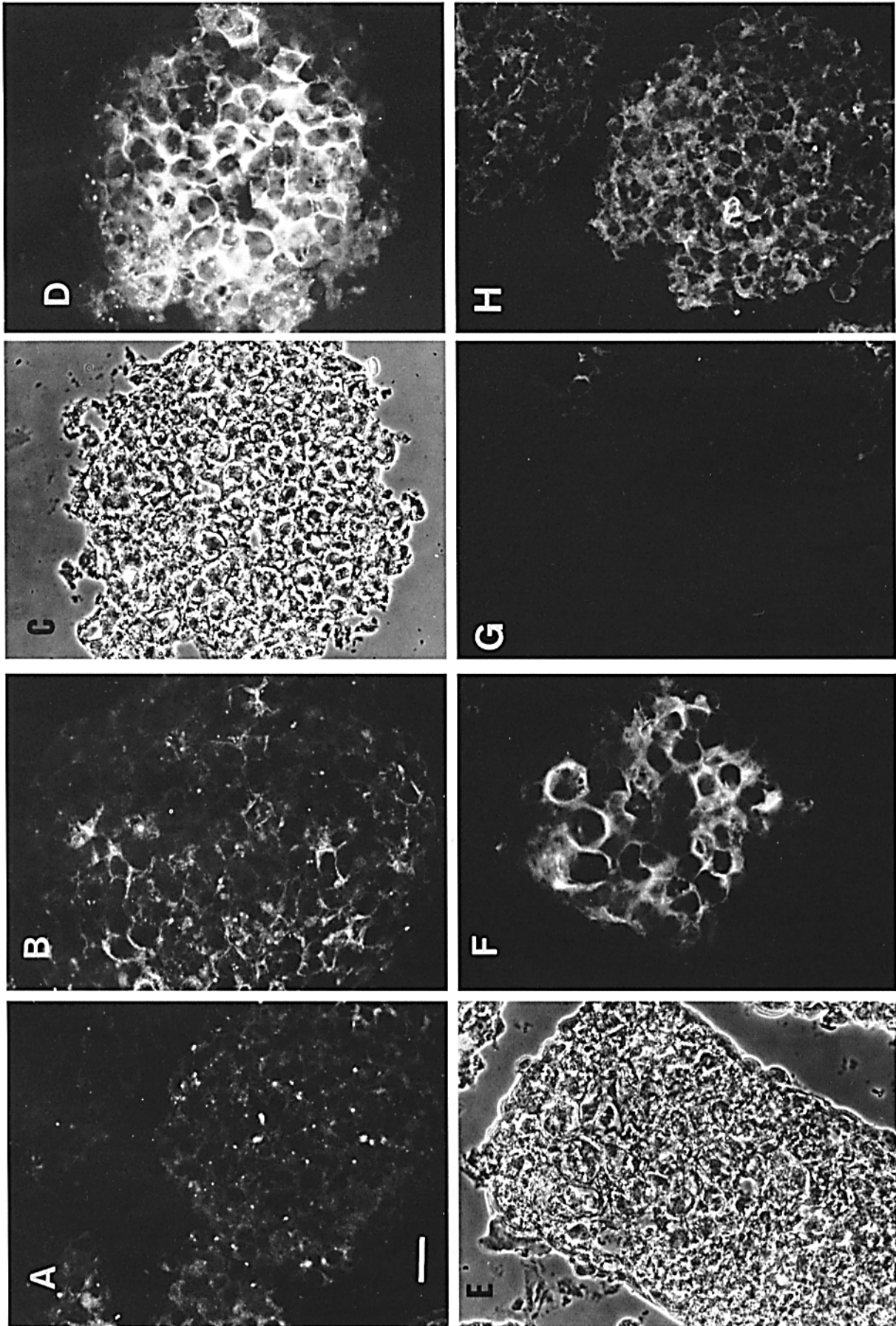


Figure 6. Indirect immunofluorescence labeling with specific anti-type II Col (*A-D*) or anti-proteoglycan antibodies (*E-H*) or anti-type I Col antibodies (*G-H*) on nodule sections. (*A*) Intracellular staining in aggregated cells at day 0. (*B*) Weak type II Col secretion in nodules at day 25; a similar staining is obtained with specific anti-type I Col antibodies. (*C-D*) Intense type II Col secretion at day 50 and phase-contrast micrograph of the same field. (*E-F*) Proteoglycan secretion in a condensed area of a nodule at day 50 and phase-contrast micrograph of the same field. (*G*) Lack of type I Col secretion at day 50. (*H*) De novo type I Col secretion in a nodule at day 85. Bars: (*E* and *F*) 30 μ m; (*G*) 10 μ m; (*H*) 30 μ m.

of C1 Cells. Northern blots were performed to characterize expression of adipocyte-specific genes during commitment of C1 mesodermal cells towards adipocyte differentiation. LPL and A2COL6 transcripts, two known early markers of adipose cell differentiation, are not detectable as long as C1 cells have not established contacts (Fig. 5). At confluency or in 3D aggregates, A2COL6 and LPL mRNAs are expressed, independent of the presence or absence of any inducer (Fig. 5). The level of these transcripts remains similar during adipocyte differentiation (Fig. 7).

After day 14, lipid droplets appear in C1 cells and the transcripts encoding the ALPB or the GPDH start to accumulate (Fig. 7). If one of the inducers (insulin or DEX) is missing, ALBP and GPDH transcripts are not detected. This behavior is in agreement with the fact that adipocytes are never observed without inducers or treatment by insulin alone. Upon long term exposure (50 d) to DEX alone, less than 20% of C1 cells show lipid droplets. As expected,

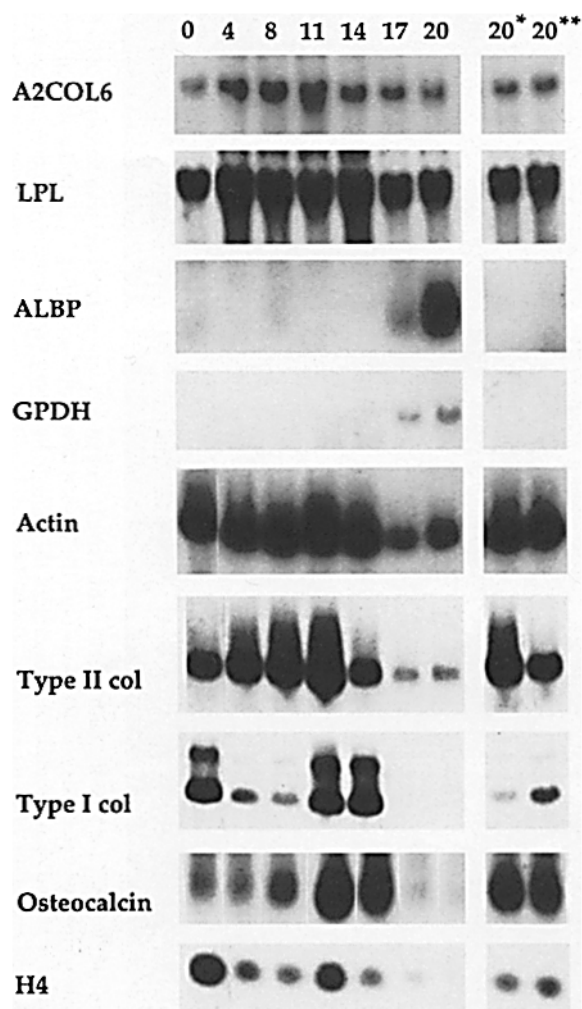


Figure 7. Kinetics of gene expression during adipogenic differentiation analyzed by Northern blotting. α_2 chain of type VI collagen (A₂Col6); lipoprotein lipase (LPL); adipocyte lipid-binding protein (ALBP); glycerol-3-phosphate dehydrogenase (GPDH); type II Col; type I Col; Osteocalcin; Histone H4; β actin. Total RNA were extracted from cells treated for 0, 4, 8, 11, 14, 17, or 20 d by DEX and insulin or extracted from C1 cells treated for 20 d with DEX(20*) or insulin alone (20**).

the level of actin mRNA decreases concomitantly with adipose cell differentiation (Cornelius et al., 1994).

Noteworthy, the steady-state levels of type I and II Col and osteocalcin transcripts decrease concomitantly with the acquisition of late markers of adipogenesis (day 17) (Fig. 7). At the same time, the cells stop dividing and more than 80% become irreversibly engaged towards the terminal differentiation. The absence of cartilage or bone matrix formation during adipogenesis is further demonstrated, in long term cultures (50 days), by the lack of staining with alcian blue or Von Kossa after addition of β GP (not shown).

Lipolytic Property of C1 Adipocytes. One of the most important characteristics of adipocytes is their ability to respond to β -adrenergic agonists by the hydrolysis of stored triglycerides. To analyze the lipolytic properties of C1 adipocytes, we pre-labeled the endogenous triglycerides on the fatty acid moiety using [¹⁴C]acetate. The addition of isoproterenol results in a strong fatty acid release which remains nearly linear for at least 2 h (Fig. 8 A). The level of fatty acid release at 2 h varies with the concentration of isoproterenol (Fig. 8 B). A maximum 10-fold increase is obtained with the addition of 1 nM isoproterenol. The half-maximal effective concentration is \sim 50 pM, thus showing the high sensitivity of C1 adipocytes to this agent. The β -adrenergic antagonist, propranolol (1 μ M) partially prevents the isoproterenol-induced lipolysis (Fig. 8 B). These results suggest that β -adrenergic receptors mediate the lipolytic action of isoproterenol. Finally, insulin exerts an antilipolytic effect on differentiated C1 cells (Fig. 8 B). Thus, these cells clearly express the lipolytic properties characteristic of adipocytes.

Transition to Terminal Chondrogenic or Adipogenic Differentiation Is Accompanied by the Loss of the Proliferative Potential of Immortalized C1 Cells. While immortalized by SV40T antigen under the control of adenovirus E1A promoter, the clonal C1 cells have retained the capacity to respond to signals and to convert into homogenous differentiated progeny along mutually exclusive pathways. To gain some insight in the balance between proliferation and differentiation of these cells, their proliferative state was evaluated using histone H4 gene expression as an index of DNA replication.

C1 cells continue to proliferate during the time course of the three differentiation programs. The level of H4 transcripts drops sharply only: (a) at day 85 of the chondrogenic differentiation, when large foci of hypertrophic chondrocytes have expanded within the C1 nodules (Fig. 3) (b) at day 17 of the adipogenic differentiation, when detectable lipid droplets accumulate in the cytoplasm (Fig. 7). In the case of the osteogenic differentiation, [³H]thymidine incorporation experiments had already indicated a block in the proliferation of the osteocyte-like cells, as soon as they are embedded within the calcified matrix (Chentoufi et al., 1993). Therefore, while maintaining C1 cells in their proliferative state along each differentiation program, the expression of SV40 T antigen does not prevent an arrest of the cell cycle when the terminal stages of differentiation are reached.

Discussion

This study presents the teratocarcinoma-derived C1 clone

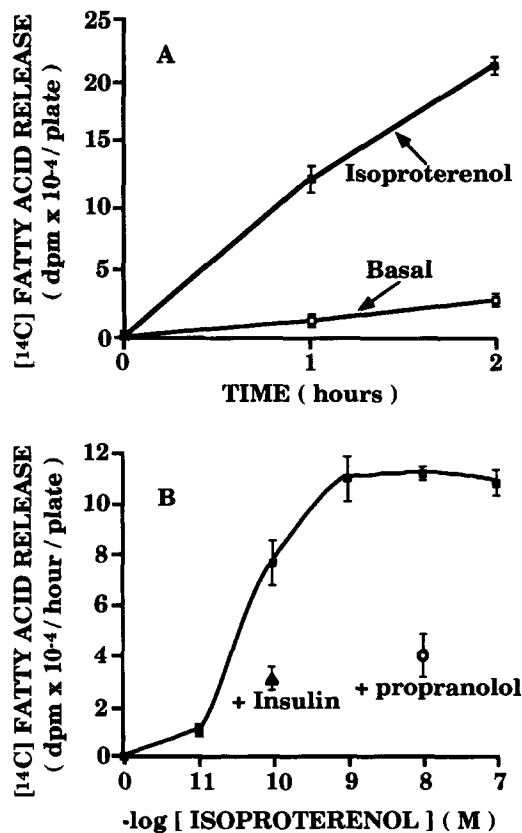


Figure 8. Lipolytic response of differentiated C1 cells to isoproterenol. (A) Time course of isoproterenol action □ basal; ■ isoproterenol 10^{-7} M. (B) Effect of isoproterenol concentration. Lipolysis was carried out for 1 h with increasing concentration of isoproterenol in the absence ■ or in the presence ○ of 10^{-6} M propranolol; ▲ cells were preincubated for 30 min with 10^{-7} M insulin, and then isoproterenol 10^{-10} M was added for 1 h. Fatty acid-linked radioactivity present in the medium at time 0 was subtracted from each value. Values represent the mean \pm SEM data obtained from six plates in two independent experiments.

as a mesodermal stem cell (a) having the capacity of self renewal by maintaining a stable undifferentiated phenotype under long term standard culture conditions, and (b) giving rise upon induction to a progeny which can be committed to three mesodermal fates: bone, cartilage, or adipocyte. C1 cells are driven towards terminal stages of each alternate pathway with high frequency, depending only on two sequential types of epigenetic signals. The first signal is mediated by the establishment of contacts between the cells and the second by the addition of appropriate inducers.

The Chondrogenic Differentiation of C1 Cells Is Similar to In Vivo Chondrogenesis

The factors regulating chondrogenic differentiation are still largely unknown, partly because of the lack of inducible model systems. Moreover, in vitro, chondrocytes rapidly lose their phenotypic markers such as type II Col and cartilage proteoglycan (Mayne et al., 1976; von der Mark et al., 1977). Immortalized clones retaining constitutive type II Col expression have been isolated from rat calvaria (Grigoriadis et al., 1989), *c-fos*-induced cartilage tumors

of chimeric mice (Wang et al., 1993) or from embryonic mouse limb chondrocytes infected by a recombinant retrovirus transducing the SV40 T antigen (Mallein-Guerin and Olsen, 1993). The C1 clone corresponds to an earlier state of commitment. Its potential for proliferation and the homogeneity of its phenotypic conversion offer, therefore, the opportunity to follow the temporal sequence of events underlying in vitro chondrogenesis, from the C1 progenitor cell stage to a fully differentiated culture.

The commitment of C1 to the chondrogenic pathway appears to be controlled at two levels: (a) cell-cell interactions in 3D aggregates provide the permissive condensed environment for cartilage differentiation. Transcription of type I, II, XI Col are triggered at this stage, although the matrix has not yet been formed; (b) the addition of 10^{-6} M DEX leads to time-dependent changes in gene expression during the development and maturation of cartilage foci to the hypertrophic stage. At day 40, more than 80% of the C1 aggregates have developed a profuse extracellular matrix of type II Col and proteoglycan. The effects of glucocorticoids on chondrocyte progenitors vary depending on the culture systems (Kato et al., 1985; Takano et al., 1985). As observed for the calvaria RCJ3.I cell line (Grigoriadis et al., 1988), C1 chondrocytes appear in the presence of DEX (10^{-6} M) and the frequency of differentiation decreases with a lowering of the DEX concentration (frequency $<10\%$ at 10^{-8} M). DEX is likely to act as a single cell signaling molecule. Interferences with autocrine or growth factors such as proteins members of the TGF β family (Adams and Watts, 1993; Tschan et al., 1993; Reddi, 1994) or undefined components of the media (1% FCS) may also occur.

The pattern of gene expression in chondrocytes of mesectodermal (neural crest) or diverse mesodermal lineages (somites, limb bud, notochord, etc.) has been only partly followed during embryogenesis. Nevertheless, the few available in vivo observations indicate that cartilages of different developmental fates have features in common with those observed during the kinetics of C1 in vitro chondrogenesis. At the earliest stages, the mesenchymal precursors condense and produce type I and a basal level of type II Col. In addition, they express type XI Col mRNA (Cheah et al., 1991; Nah et al., 1992). After the stage of precartilagenous condensation, cartilage cells become detectable histologically and a switch occurs in Col synthesis from type I to type II. Type I Col synthesis decreases, while the levels type II and XI Col transcripts increase (Castagnola et al., 1988; Strauss et al., 1990). Afterward, proliferating chondrocytes express all the cartilage-specific marker transcripts, namely aggrecan, link protein, type II, IX, and XI Col (Kosher et al., 1986; Bornstein and Sage, 1989). As differentiation continues towards chondrocyte hypertrophy, type X Col becomes synthesized (Taccetti et al., 1987; Bruckner et al., 1989). In the case of C1, the transcripts of all the genes encoding specific cartilage matrix components (van der Rest et al., 1993) are expressed at the stage of patent chondrogenesis (day 25). This behavior parallels the in vivo situation (Stirpe and Goetinck, 1989; Wood et al., 1991; Cheah et al., 1991; Ng et al., 1993). Type II Col and aggrecan mRNAs are present (day 25) before the corresponding proteins become profusely settled within the C1 extracellular matrix (day 30-

50). As observed in the adipogenic differentiation of the C1 cells, the steady-state level of the osteocalcin mRNA sharply drops at the time of overt expression of the cartilage phenotype (day 50). The de novo synthesis of type I Col by resting C1 chondrocytes (at day 85) are puzzling but have already been reported (Strauss et al., 1990; Descalzi-Cancedda et al., 1992). It might contribute to the potential of hypertrophic chondrocytes to prepare cartilage before ossification. Beyond the role of DEX, the development of the chondrocyte phenotype may depend also on: (a) the interactions of the cells with matrix components and (b) autocrine factors, hormones or growth factors concentrated within the matrix (Adams and Watt, 1993; Tschan et al., 1993; Reddi, 1994). Such agents added to the culture medium could improve the response of the C1 cells to DEX and reduce the time course of their differentiation.

In spite of the presence of Dex (10^{-6} M or either 10^{-7} M), islands of adipocytes were never observed during the chondrogenic differentiation unless AA was added. As observed for other murine embryonic cell lines (Mallein-Gerin et al., 1993; Atsumi et al., 1990) and for human or bovine chondrocytes (Kirsh et al., 1992; Daniel et al., 1984), AA does not seem absolutely required for the formation of the cartilagenous matrix (Fig. 2 and 6, D and F). When AA (50 μ g/ml) is added to Dex-treated aggregates, the nodules becomes larger and more adherent but the kinetics of chondrogenic differentiation is not significantly reduced and clusters of adipocytes reproducibly appear within 25 d at the periphery of the spreading aggregates. This effect of AA, as a potent activator of adipose cell differentiation was previously observed (Ono et al., 1990; Kawada et al., 1990).

The Adipogenic Differentiation of C1 Cells Resembles That of Preadipocyte Cell Lines

The C1 progenitor cells can also be committed to the adipogenic program. At confluency, addition of both DEX (10^{-7} M) and insulin (10^{-6} M) are strictly required to trigger the conversion of more than 80% of the C1 precursors to adipocytes. Since the C1 cell line has also the capacity to produce bone and cartilage, it must represent an earlier stage of developmental commitment than that of classical preadipocyte cell lines which all are unequivocally committed to adipogenesis (for review see Ailhaud, 1992).

Early and late events characterizing the differentiation in various adipogenic cell lines can be recognized in C1 cells. The mRNAs encoding the early markers LPL and

A2COL6 become detectable as soon as the cells reach confluency. The GPDH and ALBP transcripts, which mark terminal differentiation, appear at the time when lipid droplets arise from triacyl glycerol accumulation. Interestingly, C1 cells express high levels of type I and II Col as well as osteocalcin mRNAs until the onset of the late stage of adipogenesis. Since these markers are those of bone and cartilage, the question may be asked whether reversion towards an osteogenic or chondrogenic direction can be obtained during early adipogenesis. This is of obvious interest for particular skeletal connective tissue pathology in which bone loss is associated with an increase of adipose tissue (Meunier et al., 1971).

Concluding Remarks

During embryogenesis, osteogenic and chondrogenic stem cells may arise from the sclerotomal cells of the somitic mesoderm, the somitomeres of the hindbrain or the mesectodermal cells of the neural crest. They are believed to derive from a common embryonic progenitor cell (Thoroughgood, 1988; Couly et al., 1992; Recker, 1992) whereas the developmental origin of fat cells remains unclear. Primitive fat cell clusters are formed at the end of the human gestation period, whereas, in rodent, white adipose tissue cannot be detected during embryonic life or even at birth (Ailhaud et al., 1992). Nevertheless, it is generally agreed that adipose precursors arise from multipotential embryonic stem cells determined to become either adipoblasts or other types of mesodermal cells. In the adult, there is also evidence that adipogenic or osteogenic cells might derive from a common stromal stem cell of the bone marrow (Fridenstein, 1976; Beresford, 1989; Bennett et al., 1991; Berry et al., 1992). It also confirms the close ontogenic relationship occurring between various members of the skeletal connective tissue family.

Fig. 9 summarizes our current views about the developmental stages of the C1 mesodermal cell line. The clonal C1 progenitor is tripotent capable of giving rise to either osteocytes, chondrocytes, or adipocytes. In sparse cultures, it exhibits the properties of a true progenitor cell; (a) It possesses an intrinsic developmental program restricted to mesodermal lineage; (b) It has the ability of cell renewal; (c) It displays a stable immature phenotype associated with a lack of significant expression (Northern blot limited detection) of any differentiation markers. In addition, it expresses a series of genes encoding transcription factors that are potential regulators of mesodermal stem

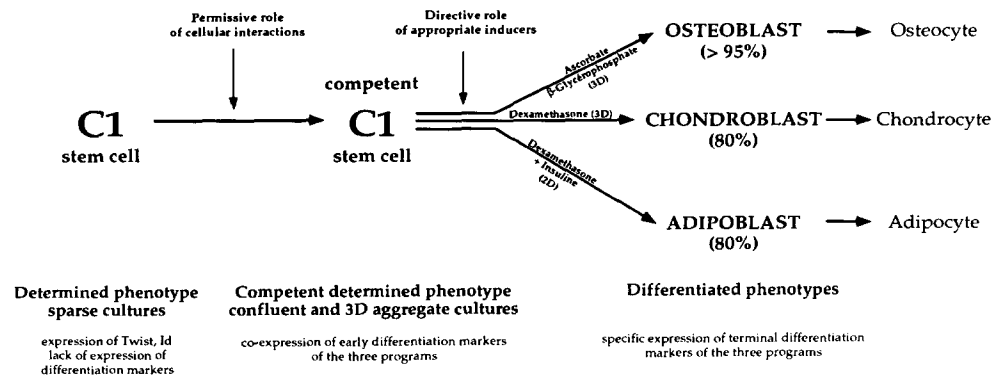


Figure 9. Our current view on the differentiation steps of the C1 stem cell.

cell fate such as Twist or Id₁ (Fig. 5), two members of the bHLH gene family known to be expressed in the early sclerotome and limb bud (Evans and O'Brien, 1993).

The establishment of cellular interactions has a critical role in C1 osteogenic differentiation (Poliard et al., 1993) and in the triggering of the transcription of early genes related to cartilage (type I, II, XI Col) (Figs. 3 and 5) or adipocyte (LPL, A2COL6) differentiation (Figs. 7 and 5). Our results suggest that the spatial organization of C1 cells, 3D or monolayer cultures is important to provide a permissive environment for differentiation to take place. The C1 progenitor cells become competent to respond to directive signals eliciting alternative fates, provided they have established appropriate contacts. The competent C1 cells are still only "determined," since they do not differentiate spontaneously. In vivo, interactions between homologous cells appear to play a fundamental role in the initiation of mesodermal programs and the control of gene expression (Gurdon, 1988). Indeed, prechondrogenic and preosteogenic condensations are a prerequisite for skeletal formation (Daniels and Solursh, 1991) and primitive fat cluster formation precedes the development of adipose tissue (Ailhaud et al., 1992). Little is still known of the precise role of cadherins, integrins, or growth factor receptors in the commitment of mesodermal precursor cells (Takheichi, 1988) but these molecules certainly appear as first choice candidates in the mediation of these interactions. In particular, members of the TGF β superfamily of growth factors such as activin, TGF β , and BMPs have been shown to be important in the processes of condensation and differentiation of cartilage, bone, and fat cells (Jiang et al., 1993; Leonard et al., 1991; Chen et al., 1991; Ahrens et al., 1993; Rosen et al., 1994). In this context, the in vitro C1 clonal system will be helpful for deciphering the role of these molecules in mesodermal differentiation.

Although systematic experiments for each differentiation marker have not yet been performed, the results from Northern blot, in situ, and immunocytochemical analyses support the idea that the great majority of the C1 cells having reached the competent state, coexpress the early genes of the three differentiation programs.

Finally, the addition of appropriate inducers is strictly required to turn on one or the other differentiation program. Each inducer acts as an instructive signal since it elicits a temporal regulation of gene expression capable to drive cells to the differentiated endstage. As compared to the other mesoblastic cell lines and in particular to the C₃H10T1/2 cell line, the C1 cells are able to univocally differentiate towards a single phenotype either chondrocyte, osteocyte, or adipocyte. These phenotypic conversions occur in each case with a very high frequency and have features close to those of the corresponding in vivo differentiation processes. Beyond the fact that C1 cells provide a valuable tool for the study of in vitro chondrogenic differentiation, the C1 clone also appears as a promising system for deciphering the molecular basis of mesoblast ontogeny.

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