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Chondroblastoma and chondromyxoid fibroma: disentangling the neoplastic chondrogenesis of two rare cartilaginous tumours

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Citation

Salvatore, R. (2010, June 22). *Chondroblastoma and chondromyxoid fibroma: disentangling the neoplastic chondrogenesis of two rare cartilaginous tumours*. Retrieved from <https://hdl.handle.net/1887/15712>

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Note: To cite this publication please use the final published version (if applicable).

Chapter 4

Chondromyxoid fibroma resembles *in vitro* chondrogenesis, but differs in expression of signalling molecules.

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Abstract

Chondromyxoid fibroma is a rare benign cartilaginous bone tumour characterized by morphological features resembling different steps of chondrogenesis, both in terms of cellular morphology - ranging from spindled to rounded cells-, as well as extra cellular matrix formed - ranging from fibrous to cartilaginous matrix. The presence of signalling molecules regulating the spatial expression of proteins involved in normal cartilage proliferation and differentiation in chondromyxoid fibroma WSS tested in samples of 20 patients and compared with articular chondrocytes cultivated in 3D pellet culture from 11 normal donors. Sections were stained with Safranin-O and HE and immunohistochemistry was performed for p16, cyclin D1, FGFR3, BCL2, p21, PTHLH, PTHR1 and N-cadherin. Expression patterns were analyzed using hierarchical clustering.

In chondromyxoid fibroma specific morphological features correlated with a distinct pattern of expression. Comparison with normal chondrocytes in pellet culture showed striking morphological resemblance, though an unmistakably different pattern of expression. N-cadherin, PTHLH and PTHR1 were significantly higher expressed ($p < 0.01$) in articular chondrocytes pellets and, conversely a significantly lower expression of Cyclin D1, p16 and BCL2 was found ($p < 0.05$). Morphological similarities reflect common steps in cartilage differentiation albeit driven by different molecular mechanisms. The proteins we have found to be differentially expressed seem crucial in neoplastic chondrogenesis.

Introduction

Chondromyxoid fibroma (CMF) is a benign cartilaginous bone tumour with a polymorphous microscopic appearance as implicated by its name, ranging from a chondroid to a myxoid and even fibrous phenotype.^{1,2} It can affect almost every osseous site, however it is found more frequently in long (mainly proximal tibia) and flat bones - the iliac bone being the most frequent (~ 25%).^{1,3,4} The distinct histological features of CMF include lobules of spindle -, or stellate-shaped cells with abundant myxoid and chondroid extracellular matrix. Differences in extracellular matrix appearance correspond to variation in proteoglycans and collagen composition and in morphology of constituting cells.⁵ The cellular areas and the matrix-rich areas - the latter being classified as either myxoid or chondroid - differ in the amount of type I and II collagen and aggrecan. Generally, in cellular areas populated with predominantly spindle shaped cells collagen type I is found,⁵ with no evidence of the presence of collagen type II, or aggrecan. Aggrecan production on the other hand is evident in the myxoid areas, where the cells are displaying a stellate morphology. Cells possessing rounded morphology and an extracellular matrix morphology and biochemical make up similar to normal cartilage (presence of aggrecan and collagen type II) characterize the chondroid regions.⁵ Morphologically this spatial difference in cellular morphology and matrix distribution has a striking parallel to the dynamic response of normal articular chondrocytes placed into monolayer culture⁶ and subsequently maintained in various high density culture systems.^{6,7} Normal (non-neoplastic) chondrocytes, cultured on standard plastic tissue culture flasks, undergo a well-documented process called de-differentiation.^{6,8} This transition is characterized by a loss of the rounded cell shape to a fibroblast-like morphology and a shift in the proportion of aggrecan (decreasing) and collagen expression (from type II to type I).⁹ It is generally recognized that this process can be reversed (re-differentiation) in appropriate conditions such as agarose,¹⁰ or in other three dimensional high-density cultures in presence of differentiation signalling molecules such as TGF- β .⁹ During this reverse process, the cells recover their rounded morphology reflected in a different pattern of organization of the actin filaments. Moreover they revert to expressing collagen type II, aggrecan and other cartilage specific genes,^{11,12} while significantly reducing collagen type I production.¹²

These processes include cell-cell and cell- extracellular matrix interactions, mainly by integrins and N-Cadherin,¹² specific extracellular matrix deposition and differentiation toward cartilage formation are driven by several signalling molecules. In particular, in the cartilage growth regulation in the epiphyseal growth plate a key-role is played by Parathyroid Hormone related Peptide (PTHrP) and Fibroblast Growth factor (FGF), and cell cycle regulators.¹³⁻¹⁵ Furthermore these signalling molecules have been shown impaired in cartilaginous tumours.¹⁶⁻¹⁸ Based on morphological similarities of cells and extracellular matrix, we hypothesized that the histological features of CMF are reflecting different steps of *in vitro* chondrogenesis: from de-differentiated/spindle shape cells to re-differentiated/round chondrocytes with parallel production of either more fibrous or cartilaginous matrix. To test this hypothesis we performed a comparative study of CMF with cultured articular chondrocytes (de-differentiated), which were pushed towards re-differentiation through a 3-D pellet culture system. We investigated the morphological spectrum of differentiation in combination with the expression pattern using immunohistochemistry for fibroblast growth factor receptor 3 (FGFR3), BCL2,

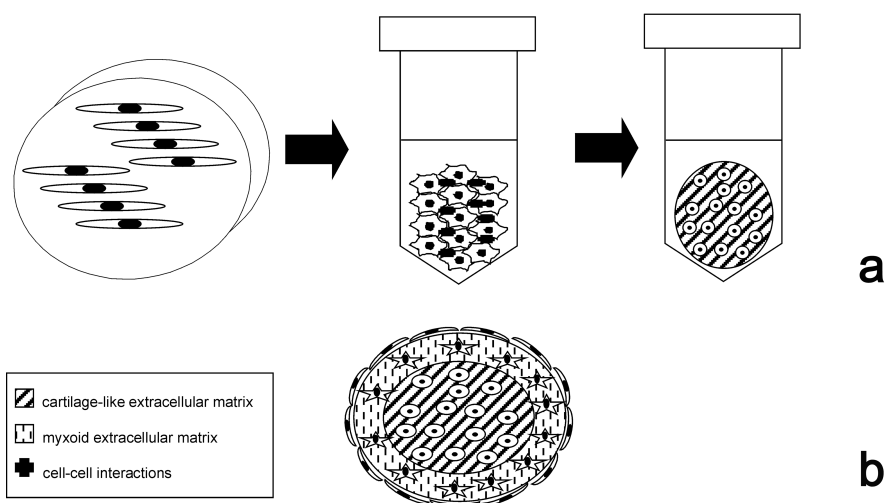


Fig.1: CMF resembles *in vitro* chondrogenesis a) Articular chondrocytes grown in flask get a spindle shape (dedifferentiation), when cultured in a 3D pellet system cells change shape and form several cell-cell and cell- extracellular matrix interaction, finally forming extracellular matrix resembling mature cartilage b) Chondromyxoid fibroma resembles *in vitro* chondrogenesis (a). The spindle cells at the periphery of the lobules morphologically resemble dedifferentiated chondrocytes (a). The lobules of myxo-chondroid matrix are similar to the cartilage formed *in vitro* (a).

p21, parathyroid hormone related peptide (PTHrP), parathyroid hormone related peptide receptor1(PTHrP1), cyclin D1, N-Cadherin and p16, in order to obtain spatial information enabling to correlate the expression profile with the morphological aspects of cells and extracellular matrix.

Methods

Pathological Material

Twenty samples of CMF were selected from eighteen primary tumour cases and two recurrent tumours. The cases were retrieved from the surgical pathology and consultation files of the Leiden University Medical Center. One primary tumour sample was kindly provided by the Department of Pathology of Ghent University. Formalin-fixed, formic acid (pH 2.1) decalcified and paraffin-embedded archival tumour tissue was available for routine staining and immunohistochemical analysis. All cases were examined following hematoxylin and eosin (HE) staining to confirm the diagnosis and Safranin-O staining to evaluate the amount of sulfated proteoglycans in extracellular matrix. All specimens were handled according to the ethical guidelines as described in the "Code for Proper Secondary Use of Human Tissue in The Netherlands", of the Dutch Federation of Medical Scientific Societies.

Articular Chondrocyte Pellets (ACP)

As described in more details elsewhere,¹⁹ cells were isolated post mortem within 24 h after death from the knee joints of a total of 11 healthy donors who specifically had no clinical history of joint disorders. These donors were selected to be of matching gender and age range. These procedures were performed in accordance

with the ethical guidelines of the Institute of Pathology, University of Bern. To create a three-dimensional environment, 0.5×10^6 cells were centrifuged at 250g for 5min in 1.5ml polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) to form a high-density pellet. The cell-pellets were maintained in culture for 2 weeks in ITS+ media (Sigma Chemical, St. Louis, USA), supplemented with TGF β 1 and dexamethasone, for a final concentration of 10ng/ml and 39.25 μ g/ml respectively.¹⁹ Cell morphology and the production of sulfated proteoglycans in extracellular matrix were examined in each pellet after 2 weeks via HE and Safranin-O staining, respectively.

Immunohistochemistry

Immunohistochemical analysis was performed on 4 μ m sections according to standard laboratory procedures.^{17,18} Details of antibodies and antigen retrieval procedures used are listed in table 2. Briefly, after pretreatment, overnight incubation with primary antibody, followed by biotin-labeled rabbit anti-mouse immunoglobulins incubation and subsequent biotinylated HRP-streptavidin complex application (DAKO, Glostrup, Denmark) were performed. Visualization was carried out in a diaminobenzidine solution (Sigma, St. Louis, MO, U.S.A.). The slides were counterstained with hematoxylin. Appropriate positive control slides were prepared according to each antibody specificity (Table 1). Moreover, internal positive controls (Table 1) were present in most of the histological slides, allowing evaluation of the antigenic property of the tissue after decalcification. As negative controls, slides were incubated with mouse or rabbit IgG of corresponding (iso-) types and concentration instead of primary specific antibodies.

The specificity of these antibodies have been validated previously and the expression

Table 1. Details of the used antibodies and immunohistochemical protocols

Antigen	Source	Clone	Staining	Positive control	Internal control	Dilution	Antigen. retrieval
Cyclin D1	Dako	MC DCS6	nuclear	tonsil	occasional endothelial cells	1:4000	mwo/10'
p16	Neomarker	MC 16 PO4	nuclear	tonsil	keratinocytes / lymphocytes	1:100	mwo/10'
FGFR3	Sigma	PC	Cytoplasm	umbelical cord	striated muscle / blood vessel walls / connective tissue/ osteoclasts	1:2000	trypsin
p21	Calbiochem	MC AB1	nuclear	colon	None	1:400	mwo/10'
PTHLH	Oncogene	PC	cytoplasm	skin	None	1:200	trypsin
PTHr1	Upstate	MC 3d1.1	cytoplasm	skin	blood vessel walls / osteoblasts	11:100	mwo/10'
BCL2	DAKO	MC 124	cytoplasm	tonsil	osteoblasts / lymphocytes	1:100	mwo/10'
N-cadherin	Transduction lab	MC 32	membranous/cytoplasm	heart	nerves	1:100	mwo/10'

PC: polyclonal; MC: monoclonal; FGFR3: fibroblast growth factor receptor-3; PTHLH: parathyroid hormone related peptide; PTHr1: parathyroid hormone related peptide

levels were compared to Q-PCR results.¹⁶

Evaluation and criteria used for scoring

The immunostained slides were assessed and scored by three pathologists independently (SR, JVMGB and PCWH) using the sum of intensity of signal (0=no expression, 1=weak expression, 2=moderate expression; 3=strong expression) and the number of positive cells (% tumour cells: 0=0%; 1=1-25%; 2=26-50%,

3=51-75% 4=76-100%) as described previously by us¹⁸ and others.²⁰ The 3 authors together, reaching a consensus, revised discrepant scores. The above mentioned scoring system emphasising both staining intensity as well as percentage of cells has been shown to be highly reproducible in our hands, and has been used in previous studies on decalcified bone tumour specimens as well.^{17,18}

The cellular areas and the matrix-rich areas were evaluated separately if they constituted at least 10% of the surface on the slide. A final weighted score, adapted from Grogan et al²¹ was calculated as the sum of score of single areas multiplied by the relative percentage extension of the area. The mean value of the sum score was reported. Finally, the cellular localization (nuclear, cytoplasmic, and membranous) of immunopositivity was noted.

Statistical analysis

Paired, two-tailed t-test was applied in order to evaluate significantly different distributions of final sum score values, between matrix-rich and cellular areas in CMF.

Unpaired two-tailed t-test, unequal variance, was applied in order to evaluate statistically significant different distribution of CMF's final weighted sum score values versus ACP's final sum score. A value of $p < .05$ was considered significant. All statistical analysis, if not differently specified, was performed by means of SPSS 10 software package. Cluster analysis was done using the data of the separate scores of intensity and the number of positive cells. One case of CMF was discarded because of too many absent values. The data were normalized, mean centered and average linkage method was applied by means of Cluster and TreeView programs.²² For similarity metrics uncentered correlation was used.

Results

Morphological and Histochemical Evaluation

Chondromyxoid fibroma: All the retrieved cases fitted the diagnostic criteria for CMF being formed by lobules of spindle or stellate shaped cells with abundant myxoid and chondroid intercellular matrix.^{1,2} In such lobules a zonal architecture could be recognized. The periphery appeared to be cellular with a low amount of extracellular matrix. More towards the center of the lobules there was more extracellular matrix with both myxoid and chondroid features, being the areas more similar to hyaline cartilage closer to the center. The transition between cellular and matrix-rich areas was not well demarcated with the two areas gradually merging together. This was reflected in a gradual change of cell shape, being slender in the cellular areas, stellate and triangular in the myxoid areas and round in the cartilage-like areas. Cells were large in the periphery and smaller towards the center. The vascular location followed a zonal architecture as well with vessel rich areas at the cellular periphery of the lobules and absence of vessel in the central cartilage-like areas

Articular chondrocyte pellet: The pellet samples showed similar morphology to CMF both in terms of architectural pattern as well as cell cytology. Rounded cells intermingled with stellate cells were present in most of the pellets, together with abundant extracellular matrix characterized by myxoid and chondroid features. Spindle cells were present mainly in a narrow area at the periphery just behind the surface (Fig. 2E). A striking similarity between the morphological features of

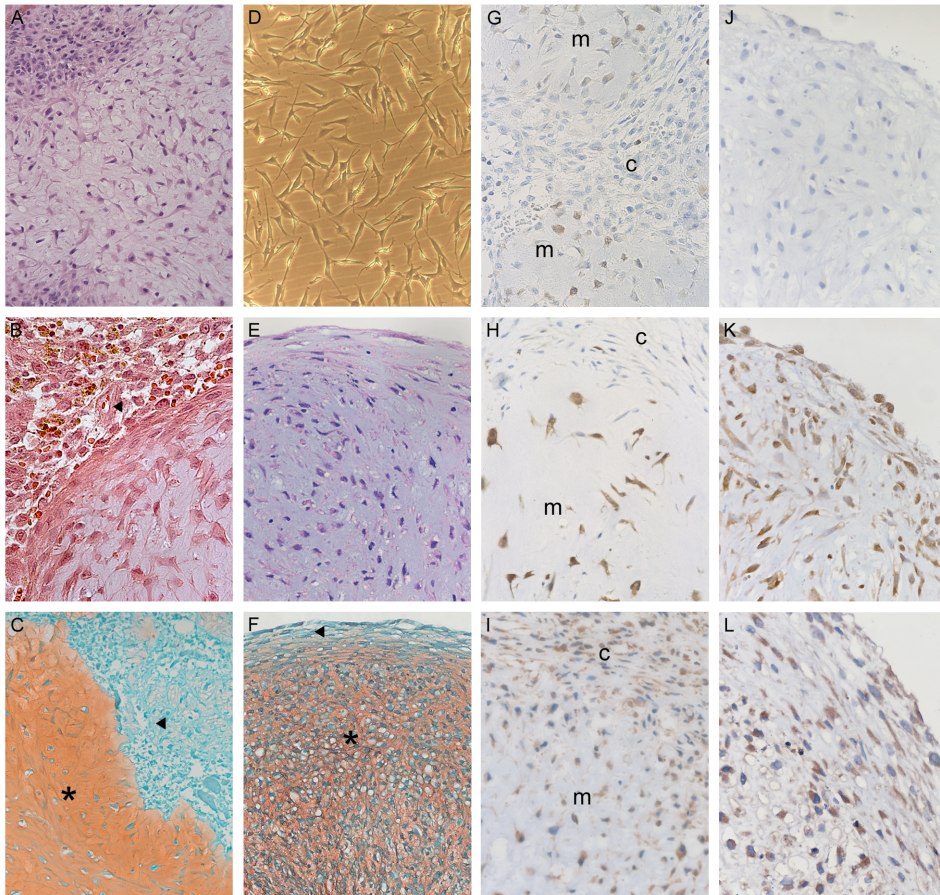


Fig 2: Morphological similarities between CMF and *in vitro* chondrogenesis (from A to F). Left column CMF, right column articular chondrocytes; A) spindle and stellate cells in the myxoid areas of CMF (HE stain 40X, original magnification), B) architectural organization of CMF lobules, vessels are present at the periphery (arrow head) where cells are spindle and with low interposed extracellular matrix (cellular areas), more to the centre cells get rounder and extracellular matrix is more abundant (matrix-rich areas) (HE stain, 40X original magnification), C) the architectural organization is substantiated by the Safranin-O stain pattern, negative in the cellular areas (arrow head), positive in the matrix-rich areas (*) (20X original magnification) D) articular chondrocytes grown in monolayer loose their round shape and become spindle or stellate (inverted microscope, no stain, 40X original magnification), E) Articular chondrocyte cultivated in 3D pellet show spindle shape at the periphery with low amount of extracellular matrix more to the centre cells get rounder and extracellular matrix is more abundant (HE stain, 40X original magnification), F) the ECM of the periphery of the pellet is negative for Safranin-O stain (arrow head), while the center whereas cells are rounder is positive (*) (20X original magnification) Expression pattern in CMF and *in vitro* chondrogenesis (from G to L): left column CMF, right column ACP; G) BCL2 immunostaining is present in CMF and higher in matrix-rich area (M) versus cellular (C), H) PTHLH immunostaining in CMF shows significantly higher expression in matrix rich area (m) versus cellular (c), I) N-cadherin immunostaining in CMF shows significantly higher expression in cellular areas, J) BCL2 immunostaining is absent in ACP, K) PTHLH immunostaining in ACP shows significantly higher of expression than CMF (40x original magnification)L)

spindle and stellate cells of CMF and the articular chondrocytes cultivated in monolayer was evident (Fig. 2A and D). In both pellets and CMF's lobules Safranin-O staining substantiated the morphologically observed pattern. Areas with intense glycosaminoglycan staining were present at the center of the lobules of CMF and throughout the ACP, while the peripheral areas showed no positive stain at all (Fig. 2C and F). Areas with mild stain showed myxoid appearance of the extra cellular matrix and stellate shape of the cells.

Immunohistochemistry evaluation

The results are summarized in table 2.

Chondromyxoid fibroma: In CMF cases the level of immunoreactivity was generally higher in the matrix-rich areas versus the cellular, being significantly higher ($p < 0.05$) for p21, Cyclin D1 and PTHLH. For N-cadherin a significant ($p < 0.001$) higher expression in the cellular areas was observed (Fig 2 G, H and I).

Articular chondrocyte pellet: Noteworthy was the absence of stain for BCL2 in all ACP (Fig 2J). Due to the narrow extension of the cellular/ peripheral areas ($< 10\%$ of the surface on the slide), it was not possible to score cellular/peripheral areas versus central/matrix-rich areas separately as for CMF. Hence only a general score was performed. The comparison between CMF and pellet showed differences in expression: a significantly ($p < 0.05$) higher expression for p16, Cyclin D1 and BCL2 (Fig 2G and J) was found in CMF versus ACP, with BCL2 being completely

Table 2. Semi-quantitative scoring results specified for cellular or matrix-rich area

Antigen	CMF C.A.	CMF M.A.	t test	CMF W.S.	ACP	t test
CYCD1	2.6±1.1	3.4±1.1 ^a	<0.01 ^b	3.2±1 ^a	1.9±1.5	0.02 ^b
p16	3.9± 1.3	4.1±1.4	0.8	4.1±1.3 ^a	2.2±1.2	<0.01 ^b
FGFR3	6.7±0.5	6.6±0.6	0.3	6.7±0.5	6.4±1.1	0.5
p21	2.8±1.1	3.4±1.1 ^a	0.04 ^b	3.2±1.1	3.2±1.5	0.9
PTHLH	2.3±1	4.3±1.4 ^a	<0.001 ^b	4±1.4	6.8±0.4 ^a	<0.001 ^b
PTHR1	5.7±1.1	5.5±0.9	0.8	5.2±1.6	6.5±0.5 ^a	<0.01 ^b
Bcl2	3.1±1.5	3.6±1.5	0.3	3.6±1.5 ^a	0±0.0	<0.001 ^b
N-cadherin	5.3±1.4	4.3±1.3	<0.001 ^b	4.9±1	6.9±0.3 ^a	<0.001 ^b

Values are reported as mean sum score ± standard deviation, a significantly higher mean sum score and b significant p values. C.A.: cellular areas, M.A.: matrix-rich areas, W.S.: weighted score.

absent in ACP. Conversely a significant ($p < 0.05$) higher expression of N-Cadherin (Fig 2I and L), PTHLH (Fig 2H and K) and PTHR1 was found in ACP versus CMF. Generally the results of significance was the same either assuming equal or unequal

variance. The different pattern of expression resulted in the 2 clusters visualized by the hierarchical clustering analysis (Fig 3).

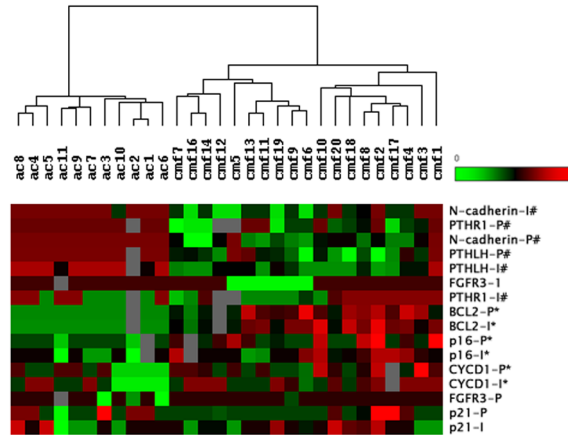


Fig 3: Hierarchical clustering. 2 different clusters are evident (the tree in the upper part of the figure, on the left side the articular chondrocytes: AC; on the right side the cases of chondromyxoid fibromas: CMF). As exemplified by the bar on the right side, in the rest of the figure (heat map) the green color blocks represent low value and red color blocks high value of the studied proteins (I: intensity, P: percentage). Colors in between are intermediate, with black being the mean value; gray blocks represent missing data. #: proteins significantly higher in pellet; *: proteins significantly higher in CMF.

Discussion

The morphological spectrum of CMF, both in terms of type of extracellular matrix produced as well as the resident neoplastic cells, is broader than what is normally observed in normal mature hyaline cartilage. The observed morphological features are suggestive for the recapitulation of *in vitro* chondrogenesis, and this has prompted us to study the phenotype of the neoplastic cells, the specific extracellular matrix present and their relative profile of expression of different molecules known to be involved in cartilage differentiation. Our study showed that the expression of these regulators of cartilage differentiation and cell cycle regulatory molecules differed significantly in cellular areas versus matrix-rich areas of CMF. Most of the tested proteins were, more extensively and intensely present in the matrix-rich areas. This could reflect the role of these molecules in promoting both the deposition of abundant specific extracellular matrix as well as the typical cellular phenotype of the resident cells. In an opposite way, N-Cadherin expression was significantly higher in the cellular areas. This pattern strictly resembles the initial mesenchymal condensation in which N-Cadherin is present only in the undifferentiated precursors chondrocytes and lost after achieving the phenotype of differentiated chondrocytes.^{12,23} In this respect the higher expression of N-Cadherin in the cellular areas may reflect a role for the homophylic cellular interactions to commit undifferentiated cells towards chondrogenesis, later occurring more towards the center of the lobule, or conversely to maintain the less differentiated phenotype characteristic of the cellular areas.

We have used an *in vitro* system as a comparative model since we observed a

striking morphological resemblance between CMF and cultured articular chondrocytes. In particular the zonal architecture of CMF lobules strictly resembled that in chondrocyte pellet. A gradient of oxygen and nutrients may be responsible for this architecture since in both CMF lobules and ACP these have to diffuse from the periphery towards the center through the extracellular matrix. Cultured chondrocytes are shown to be sensitive to different oxygen tensions, in an inverted proportional way to the degree of differentiation.²⁴ In parallel in the central areas of the chondrocytes pellet and in the matrix-rich areas of CMF lobules, where the oxygen tension is expected to be lower, a rounder morphology of cells together with intense Safranin-O staining was seen, reflecting the phenotype of differentiated chondrocytes. Despite the striking morphological resemblance CMF and chondrocytes pellet showed different expression pattern (Fig. 3). In particular PTHLH and PTHR1 were significantly higher expressed in the chondrocyte pellet culture system. This could be the result of culture conditions promoting chondrogenesis, since the media used include high levels of TGF β 1 (10ng/ml). It is well known that this signalling molecule can induce, specifically in articular chondrocytes, up regulation of PTHLH.²⁵ However it is also known that *in vivo*, in proliferative and prehypertrophic chondrocytes of the growth plate, PTHLH binding to its receptor PTHR1 leads to the up regulation of BCL2.²⁶ Such effect is not present *in vitro* in the absence of extra doses of PTHLH.²⁶ In this regard, our results on pellets resembled the results of previous experiments,²⁶ since chondrocytes cultured in a high-density system, in the absence of supplemented extra dose of PTHLH, do not express BCL2.

The diffuse positive signal for BCL2 in CMF is, noteworthy, especially considering its lower level of PTHLH. This striking difference in BCL2 expression between CMF and the *in vitro* condition of normal chondrocytes, indicates a different mechanism of signalling/transduction, that may be due to the effect of other mediators present *in vivo* and not *in vitro*,²⁶ or the result of differences of cartilage differentiation in neoplasia versus normal cells.

An intriguing result was the different levels of expression found for p16 and Cyclin D1. These two molecules counteract in regulating cell cycle progression. Cyclin D/Cyclin dependent kinases complex phosphorylates Rb (retinoblastoma) proteins, promoting the progression of the cell cycle.²⁷ This action is counterbalanced by the binding of p16 to the complex, which in turn induces an allosteric change in Cdk4/6 thereby altering the binding site of D-type cyclins and reducing its affinity for ATP, hence inhibiting cell cycle progression.²⁷ The presence of both counteracting proteins is in agreement with the clinically benign nature of CMF. In a previous study²⁸ the expression of p16 in enchondromas and loss of expression in conventional chondrosarcomas was observed, which illustrates the role of this molecule in balancing proliferation activity typical of malignant transformation. The lower level of Cyclin D1 in ACP is of note, considering its higher level of PTHLH and the stimulation from external TGF β 1, both known to upregulate Cyclin D1 expression.²⁹ Again this result underlines the difference in signalling/transduction mechanisms between *in vitro* chondrogenesis and CMF, despite their histological similarities. The difference in N-cadherin expression in CMF versus ACP reflects the different spatial distribution: the expression in CMF is higher in the cellular areas, while in ACP is largely expressed also where cells are embedded in abundant extracellular matrix. Since the ACP were cultured for only 2 weeks, N-cadherin may still be present at a high level as the condensation phase is perhaps still

going through its end stages. We showed FGF signalling to be conserved in CMF and ACP, since the expression of FGFR3 and p21 did not differ significantly. Both molecules are part of the FGF signalling pathways in which, in chondrocytes, p21 is the downstream molecule of FGFR3 activation resulting in inhibition of cell cycle progression and indirectly promoting differentiation.¹³

The comparison between *in vitro* and *in vivo* conditions is in generally problematic. Despite the *in vitro* results of the present study are obtained under the influence of TGF- β 1, the possible influence of this condition has been extensively analyzed in the discussion. Furthermore recent data showed a diffuse presence of functionally active TGF- β 1 in CMF.³⁰

In conclusion we clearly identified and substantiated the morphological similarities between CMF and *in vitro* cell culture chondrogenesis. Similarities include cellular morphology, quality of the extracellular matrix and cyto-architecture. In our opinion this clearly reflects a conservation of basic process of cartilage formation in this neoplastic condition, further confirmed by the expression of PTHLH as well as FGF signalling molecules. The observed difference in expression of these molecules, between the matrix rich areas versus the cellular areas of CMF, may reflect the importance of these molecules in the commitment of neoplastic cells toward cartilage differentiation. The comparisons with ACP showed significantly higher expression of N-cadherin, PTHLH and PTHR1 and conversely lower expression of Cyclin D1 and p16 in ACP versus CMF. The absence of BCL2 expression in ACP is noteworthy. These differences in expression may be crucial in neoplastic chondrogenesis.

Acknowledgements

We would like to acknowledge Dr. R. Forsyth for the case from Ghent University, I. Briaire-de Bruijn and A. Yavas, for expert technical help, L. Rozeman, M.Sc. and M. Lombaerts, PhD for critical discussions. The collaborative efforts with Drs Ivan Martin and Andrea Barbero (University of Basel, Switzerland) are acknowledged.

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