REVIEW ARTICLE

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Differential gene expression in the perichondrium and cartilage of the neonatal mouse temporomandibular joint

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Structured Abstract

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Our goal was to discover genes differentially expressed in the perichondrium (PC) of the mandibular condylar cartilage (MCC) that might enhance regenerative medicine or orthopaedic therapies directed at the tissues of the temporomandibular joint. We used targeted gene arrays (osteogenesis, stem cell) to identify genes preferentially expressed in the PC and the cartilaginous (C) portions of the MCC in 2-day-old mice. Genes with higher expression in the PC sample related to growth factor ligand-receptor interactions [FGF-13 (6.4×), FGF-18 (4×), NCAM (2×); PGDF receptors, transforming growth factor (TGF)-β and IGF-1], the Notch isoforms (especially Notch 3 and 4) and their ligands or structural proteins/proteoglycans [collagen XIV (21×), collagen XVIII (4×), decorin (2.5×)]. Genes with higher expression in the C sample consisted mostly of known cartilage-specific genes [aggrecan (11×), procollagens X (33×), XI (14×), IX (4.5×), Sox 9 (4.4×) and Indian hedgehog (6.7×)]. However, the functional or structural roles of several genes that were expressed at higher levels in the PC sample are unclear [myogenic factor (Myf) 9 (9×), tooth-related genes such as tuftelin (2.5×) and dentin sialophosphoprotein (1.6×), VEGF-B (2×) and its receptors (3–4×) and sclerostin (1.7×)]. FGF, Notch and TGF-β signalling may be important regulators of MCC proliferation and differentiation; the relatively high expression of genes such as Myf6 and VEGF-B and its receptors suggests a degree of unsuspected plasticity in PC cells.

Key words: gene array; gene expression; mouse; perichondrium; temporomandibular joint

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Introduction

The mandibular condylar cartilage (MCC) first appears as a condensation of cells adjacent to the periosteum of the mandible around the 7th or 8th week *in utero*. Over the course of the next 4–6 weeks, a synovial joint develops that is the only site of articulation between the skull and jaw (except for the dentition) and also a major site of growth for the mandible. This origin of the MCC as a secondary cartilage derived from the periosteum of intramembranous bone has been well-documented in the

embryological literature (1–3) and its potential implications for the regulation of mandibular growth have been exhaustively debated in the orthodontic literature (4–7). However, attempts to exploit this peculiar developmental history for therapeutic purposes have been impaired by our relatively limited understanding of MCC cell biology. Of central importance are the cells of the prechondroblastic layer deep within the perichondrium (PC), as they (and not the differentiated chondrocytes as in a growth plate) are the locus of nearly all cell divisions in the MCC (8–10).

One of the earliest investigations of the properties of these cells was performed by Stutzmann and Petrovic (11), who published numerous studies supporting the view that orthopaedic appliances that altered the postural position of the mandible could stimulate proliferation in MCC prechondroblastic cells leading to increased growth in mandibular length and height (4, 12, 13). They postulated that the prechondroblastic zone contained cells in two stages of differentiation: an elongated 'stem-cell' type called a 'skeletoblast' which divides infrequently and a 'true prechondroblast', a rounded cell that divides more frequently. They further proposed that 'skeletoblasts' were bipotent (i.e. they would normally differentiate into preosteoblasts, but could develop into 'true prechondroblasts' with appropriate biomechanical/functional stimulation), whereas 'true prechondroblasts' had only chondrogenic potential.

Although Petrovic et al. subsequently published data contrasting intracellular calcium levels and concentrations of fibronectin, transglutaminase and heparin sulphate between skeletoblasts and prechondroblasts (11), their work essentially predated the introduction of molecular biological techniques that might have permitted further investigation of prechondroblastic layer cells. Similarly, their characterization of MCC 'skeletoblasts' as 'fibroblast-like pluripotential *stem-cells* [italics mine] derived from the embryonic mesenchymal cell' (13) has lost operationality in the succeeding decades of sophisticated applications of embryonic and adult stem-cell populations for regenerative medicine. Therefore, their seminal work left important questions unanswered: are a subset of the cells of the prechondroblastic layer 'true' stem cells or something else? If not, how differentiated are they? Although they have repeatedly been shown to be bipotent, are they pluripotent? What factors are of importance for regulating their proliferation and differentiation?

Cell culture could be a powerful tool for exploring the potential of prechondroblastic cells from the MCC, but the heterogeneity of cell types in or adjacent to the MCC (fibroblasts, prechondroblasts, non-hypertrophic and hypertrophic chondrocytes, osteoblasts/osteoclasts) has proven a challenge to obtaining a relatively homogeneous culture of prechondroblastic cells. A recurrent theme in these attempts has been the diversity of cell types in the resulting cultures derived from postnatal rodent, rabbit or primate MCC (14–16). Moreover, most efforts have first removed the PC by mechanical dissection or enzymatic digestion to focus on the chondrocytes. The closest attempt to study the prechondroblastic cells in isolation was an explant culture of the prechondroblastic layer isolated from neonatal mice MCC (17), but this study was structural rather than biochemical or molecular in nature. Numerous studies have employed explant culture of MCC with or without attached mandibles (18–24), but this approach limits the cellular/molecular techniques that can be utilized.

Despite these impediments, several studies over the last decade using a variety of experimental approaches and transgenic animal strains have begun to better define the lineage of prechondroblastic cells and to illuminate potential regulatory genes. Careful study of the developing MCC in rodents has revealed that the future condyle develops from a condensation of alkaline phosphatase-positive cells that are continuous anteriorly with the alkaline phosphatase-positive periosteum of the mandible (25). This suggests that these cells are not truly mesenchymal in character, but have already differentiated into periosteum-like cells that may still be bipotent between osteogenic and chondrogenic lineages, as proposed by Petrovic et al. (4). In the developing MCC, the bipotentiality of prechondroblastic cells is exemplified by their expression of both mRNA for osteogenic lineage markers, such as type I collagen, Runx2, Osterix and mRNA for *Sox 9*, a marker for chondrogenic differentiation (26). Thus, the MCC appears to arise from a periosteum, albeit an 'immature' one, and that periosteum can be transformed into a PC under some circumstances. Notch1 and Twist, known as cell fate mediators in a variety of tissues, are both expressed largely in the prechondroblastic layer in the developing MCC (27, 28) and expression levels of these factors may also play a role in the differentiation pathway.

Although prechondroblastic cells are bipotent, it is perhaps not surprising that their osteogenic lineage is

primary in light of their periosteal derivation. Experiments in secondary cartilage on the intramembranous bones of the chick suggest that movement/articulation is necessary for diverting the otherwise osteogenic precursors to chondrogenesis (29). This osteogenic bias is further evidenced by the fact that mice genetically altered so as not to express the osteogenic lineage precursor Runx2 do not develop a MCC (30). Viewed in this context, prechondroblastic cells of the MCC are clearly not 'stem cell-like' in the current usage of this term. They represent pre-osteogenic cells diverted to chondrogenesis in the region of articulation between two bones. However, we know relatively little about differences in gene expression between this periosteum turned PC and the underlying cartilage layers.

The goal of this study was to identify genes that are differentially expressed in the PC or cartilaginous (C) portions of the developing MCC to guide future studies of growth regulation and tissue regeneration. Although limited comparisons of gene expression have been performed contrasting cell layers in the growth plate (31) or intersutural tissue from different sutures (32), to our knowledge no investigation of this sort has been attempted for different zones of the MCC.

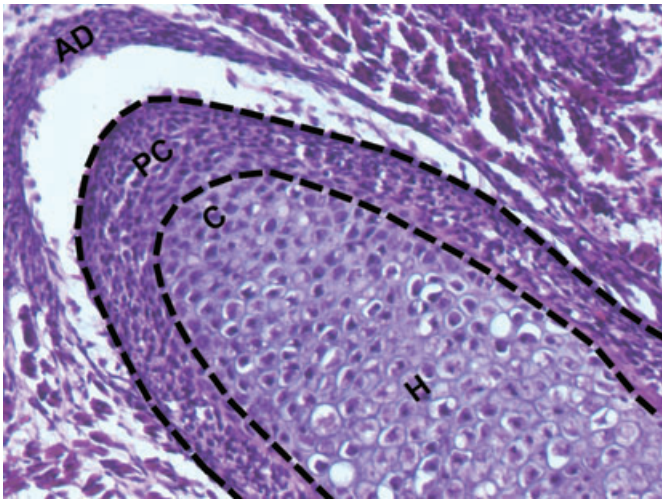

Fig. 1. Coronal section of embryonic day 18 (E18) mouse mandibular condylar cartilages illustrating perichondrial (PC) and cartilaginous (C) portions of the tissue. Dashed lines delimit tissue removed for the PC sample. AD, articular disc. Haematoxylin and eosin, 20×.

Materials and methods

The mandibular condyle and adjacent ramus were dissected from 2-day-old CD-1 mouse pups. This age was chosen because the MCC was larger than in late embryonic stage pups, but still permitted the PC to be removed with relative ease. Under a dissecting microscope, the PC was gently teased away from the underlying cartilage (Fig. 1) and the cartilage (C) was separated from the bone. The PC and C samples were then snap frozen in liquid nitrogen. RNA was extracted from pooled samples of around 50 tissues using the RNEasy Micro RNA Isolation Kit (Qiagen, Valencia, CA, USA). The quantity and quality of mRNA were measured by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

The RNA samples were then analysed using the Mouse Osteogenesis RT^2 Profiler™ PCR Array (PAMM-026; SABiosciences Corp., Frederick, MD, USA), which profiles the expression of 84 genes related to osteogenic differentiation. In a separate experiment, additional PC and MC samples were analysed using the Mouse Stem Cell RT^2 Profiler™ PCR Array (PAMM-405, SABiosciences Corp.), which profiles the expression of 84 genes related to the identification, growth and differentiation of stem cells. Genes were considered to be differentially expressed if they were expressed at least 1.5× higher in either the PC or C sample.

Results

The Osteogenesis and Stem Cell arrays identified 22 and 26 genes, respectively, that showed higher expression in the PC sample relative to the C sample (Tables 1 and 2). The highest expression was noted for type XIV collagen (21×), myogenic factor (Myf) 6 (9×), fibroblast growth factor (FGF)-13 or (6.4×), followed by several genes in the 3–4× range (collagens IV, VIII and XVIII; Notch 3 and 4; cadherins 9, 13 and 15). The Osteogenesis and Stem Cell arrays identified 13 and 20 genes, respectively, that showed higher expression in the C sample relative to the PC sample (Tables 3 and 4). The highest expression was noted for types XI and X procollagen (14× and 33×), aggrecan (11×), bone morphogenetic protein (BMP) 7 and 8 (8× and 10×), Indian hedgehog (6.7×), matrix metalloproteinase (MMP) 13 (5.9×) and osteopontin (5.3×), followed by several genes in the 3–4× range (procollagen IX, Sox 9, MMP 9 and vitamin D receptor). Most of these genes are characteristic of cartilage as a tissue or typically expressed at high levels in cartilage. Other genes that were

Table 1. **Genes with higher expression in the perichondrium identified by SuperArray™ Osteogenesis Array**

Gene	Ratio	Gene	Ratio
Alkaline phosphatase 2	1.7	Growth differentiation factor 10	2.0
Bigylcan	1.5	Insulin-like growth factor 1	1.9
BMP-4	1.8	Matrix metalloproteinase 2	1.6
Procollagen XIV	20.6	Sclerostin	1.7
Procollagen XVIII	3.6	SPARC	1.5
Procollagen IV	4.1	Tuftelin interacting protein 11	1.6
Procollagen VI	1.5	TGF-β3	1.9
Procollagen VIII	3.3	TGF-βr2	2.6
Decorin	2.4	Tuftelin 1	2.5
Dentin sialophosphoprotein	1.6	Vascular cell adhesion molecule 1	2.1
FMS-like tyrosine kinase	5.1	VEGF-B	2.1

Ratio refers to gene expression in the perichondrial sample divided by gene expression in the cartilage sample. Only ratios ≥1.5 are included.

Table 2. **Genes with higher expression in the perichondrium identified by SuperArray™ Stem Cell Array**

Gene	Ratio	Gene	Ratio
ATP-binding cassette	2.5	Kinase insert domain protein (VEGF receptor)	4.3
Actin, alpha, cardiac	5.7	Myogenic factor 5	1.5
Aldehyde dehydrogenase	4.2	Myogenic factor 6	9.1
Cadherin 13 (H-cadherin)	3.4	MYST histone acetyltransferase 4	1.8
Cadherin 15 (M-cadherin)	4.4	Neural cell adhesion molecule 1 (NCAM)	1.8
Cadherin 9 (T-cadherin)	4.7	Notch 1	1.6
Delta-like 3	2.8	Notch 3	3.5
FGF-7	1.8	Notch 4	4.1
FGF-13	6.4	Platelet-derived growth factor receptor	2.4
FGF-18	3.8	Peroxisome proliferator activated receptor-gamma (PPAR-γ)	2.7
Flt 1 (VEGF receptor)	2.7	Pre-T cell antigen receptor	1.7
Insulin-like growth factor 1	1.6	RAS-related C3 botulinum	1.7
Jagged 1	1.7		

Table 3. **Genes with higher expression in the cartilage identified by SuperArray™ Osteogenesis Array**

Gene	Ratio
BMP-7	6.7
BMP-8	5.3
Integrin binding sialoprotein	4.0
Insulin-like growth factor 1 receptor	2.2
Matrix metalloproteinase 9	3.4
Matrix metalloproteinase 13	5.9
Procollagen IX	4.5
Procollagen X	33.3
Procollagen XI	14.3
Scavenger receptor class	2.9
Sox 9	4.3
Osteopontin	3.4
Vitamin D receptor	3.7

Ratio refers to gene expression in the cartilage sample divided by gene expression in the PC sample.

Table 4. **Genes with higher expression in the cartilage identified by SuperArray™ Stem Cell Array**

Gene	Ratio	Gene	Ratio
Aggrecan	11.1	Indian hedgehog	6.7
BMP-7	8.3	Procollagen X	25.0
BMP-8	10.0	Ras homologue gene family	2.1
BMP-binding endothelial	1.6	S100 protein, beta polypeptide	2.0
Cyclin A2	1.6	Snail 1	3.3
Cyclin D2	2.2	Snail 2	1.6
Cadherin 2 (N-cadherin)	3.1	Osteopontin	5.3
Cadherin 6	1.5	Staufen (RNA1 binding protein)	2.0
Cyclin-dependent kinase 2b	4.8	Tubulin beta 3	3.7
Frizzled homologue 1	2.6	Wnt inhibitory factor 1	4.8

expressed higher in the C sample at levels between 3 and 5× included Wnt inhibitory factor 1 or WIF1, tubulin beta-3, snail 1, frizzled homologue 1, cadherin 2 and bone sialoprotein.

Discussion

In the C sample, the high expression of genes often highly expressed in cartilage can be viewed as a

'positive control' for the dissection procedure. In particular, the expression of genes such as collagen X and aggrecan at very high levels (33× and 11×, respectively) in the MC sample suggests that the tissue harvest was fairly accurate in separating cartilage from PC. Evidence that our technique was replicable is provided by the similarity of expression levels in those genes present in both arrays: BMP-7 (6.7× in Osteogenesis Array, 8.3× in Stem Cell Array), BMP-8 (5.3×, 10×), insulin-like growth factor (IGF)-1 (1.9×, 1.6×), osteopontin (3.4×, 5.3×) and procollagen X (33×, 25×).

Genes with higher expression in the PC sample

Some of the genes with higher expression in the PC sample have antecedents in the literature or fit with other observations. In other instances, their functional importance requires further investigation, while in still other cases the higher-expressed genes were unexpected. These genes can therefore be discussed in three groups: 1) genes that may be mediators of proliferation and differentiation of prechondroblastic cells; 2) genes for structural and adhesion proteins that are plausibly linked to the architecture and cell communication in the PC; 3) unexpected genes for which a ready explanation is elusive.

Potential mediators of proliferation and differentiation

This group includes the FGF isoforms and other receptors [platelet-derived growth factor receptor (PDGFr), IGF-1r, Notch 1, 3, and 4]. Three FGF isoforms were enriched in the PC sample: FGF-13 (6.4×), FGF-18 (4×) and FGF-7 (1.8×). In limb bones, FGF-18 has been localized to the periosteum, where it inhibits chondrocyte proliferation and differentiation (33), apparently under the influence of Twist-1 (34). Because Twist-1 has been immunohistochemically localized to the prechondroblastic layer (27), FGF-18 may play a similar role in the MCC, probably signalling via Ffgr2, which is also highly expressed in periosteum and in the prechondroblastic layer of the MCC (24). Neural cell adhesion molecule (NCAM), a cell-surface glycoprotein that mediates cell–cell signalling in the nervous system, was expressed almost 2× greater in the PC sample than in the C sample. A possible explanation may relate to the recent demonstration that NCAM is a major regulator of the interaction of FGF-2 with its receptors in two fibroblast cell lines (35). NCAM, which has been reported to bind to Fgfr2 (the predominant FGF receptor sub-type in the prechondroblastic layer (24)), interferes with the binding of the FGF receptor to FGF, thereby inhibiting the cellular response to FGF.

Insulin-like growth factor-1 receptor, which was more highly expressed in the C sample, has been demonstrated using immunohistochemical techniques to be localized primarily to the chondroblastic and hypertrophic portions of the MCC (24). By contrast, its primary ligand IGF-1, somewhat higher (1.6×) in the PC sample, stimulates proliferation in the perichondrial cells of the MCC (24). Similarly, the receptor for PDGF has been localized to the prechondroblastic layer of the MCC in 1- to 10-day-old rats (36); in this study, it was enriched 2.4× compared with the PC sample. Finally, transforming growth factor-beta receptor 2 (Tgf-βr2) as well as TGF-β3 were increased 2.6 and 1.9×, respectively, in the PC. This is of great interest since Tgf-βr2 appears to regulate cell proliferation in both osteoprogenitor and chondroprogenitor cells of the developing mandible, where conditional inactivation of Tgf-βr2 also results in major defects in size and organization of the MCC (37).

Members of the Notch family of trans-membrane receptors have been implicated as cell fate mediators in many tissues (38–40). They are expressed in the early stages of chondrogenic differentiation, becoming confined to the PC as differentiation proceeds (41). Of the three isoforms of Notch that were over-expressed in MCC [plus a Notch ligand, Jagged 1(1.7×)], Notch-1 (1.6×) has been localized using immunohistochemistry to the MCC prechondroblastic layer. Moreover, inhibition of Notch reduces proliferation in MCC (28). Our results suggest that Notch-3 (3.5×) and Notch-4 (4.1×), shown to be present in limb articular cartilage (42), may be of greater importance than Notch-1 in the MCC.

Structural and adhesion proteins

Some of the other genes that had higher expression in the PC sample were structural proteins or proteoglycans. At least for procollagen XIV (21× higher in the PC sample), this may relate to interactions with type I collagen and/or small proteoglycans. Collagen XIV is

distributed preferentially in tissues containing type I collagen fibrils (43) and has been shown to bind to the small proteoglycan decorin (44), which serves to modulate cellular interactions with collagen XIV (45). Because the articular and prechondroblastic layers of the PC are rich in type I collagen (46, 47) and decorin (48), the enrichment of the PC sample in mRNA for procollagen XIV and decorin (2.4×) is explicable. Although it might be thought surprising that type I collagen expression did not differ appreciably between the PC and C samples, immunohistochemical studies of the MCC indicate noticeable type I collagen in the deeper (C) layers, especially the hypertrophic layer (47).

Still other differential gene expression between the PC and C samples involved various members of the cadherin family, molecules that facilitate cell–cell adhesion and in so doing regulate cellular activities such as differentiation (49). The PC sample was enriched (3–5×) in cadherin 9 (T-cadherin), cadherin 13 (T- or H-cadherin) and cadherin 15 (M-cadherin). The relatively high expression of cadherin 13, which is a modulator of angiogenesis (50, 51), may relate to the elevated expression of VEGF-B and its receptors in the PC sample (see below). Similarly, cadherin 15, which facilitates the differentiation of myoblasts by forming a complex with beta catenin (49, 52), may be enriched in concert with the abundant muscle differentiation factor Myf6 as outlined below.

Unexpected genes

Other matrix proteins with greater expression in the PC sample relative to the C sample are less readily understood. Tuftelin (2.5×), tuftelin interacting protein 11 (1.6×) and dentin sialophosphoprotein or dspp (1.6×) are proteins first identified in the enamel and dentin of the developing tooth (53–55). However, tuftelin and dspp have been reported in bone and other non-dental tissues (53, 56), and dspp has recently been localized immunohistochemically to the prechondroblastic layer of the MCC in very young rats (57). Nevertheless, the role of these proteins in the MCC remains to be elucidated. Similarly, vascular endothelial growth factor-B or VEGF-B, a member of a family of angiogenic agents (58), is expressed at levels twice as high in the PC sample as it is in C sample; the VEGF receptors Flt-1 (2.7×) and kinase insert domain receptor/Flk-1-KDR (4.3×) are elevated to an even greater extent in the PC sample. Although the role of VEGF-A in endochondral ossification has been well-documented (59), current knowledge of VEGF-B does not explain its enrichment, and that of its receptors, in the PC of the MCC. However, chondrocytes secrete all four members of the VEGF family, and chondrogenic stimulation by BMP-2 up-regulates VEGF-B, suggesting that it has a role in growth plate physiology (60). The enrichment of the PC sample (3×) for peroxisome proliferator activated receptor-gamma (PPAR-γ) is very interesting, since PPAR-γ is known as an adipogenic-specific transcription factor (61, 62).

Sclerostin, enriched 1.7× in the PC sample, is a product of osteocytes which antagonizes Wnt signalling in osteoblasts (63). Perhaps more pertinent to the MCC, it also has been shown to inhibit the differentiation of preosteoblastic cells (64, 65). However, perhaps the most puzzling is the ninefold enrichment of Myf6 in the PC sample. Myf6 is a transcription factor that is important in the specification and differentiation of skeletal muscle myotubes during embryogenesis (66). Although work on Myf6 has been confined almost completely to muscle, it may be significant that a related gene, Myf5 (which was 1.5× higher in PC), appears to play an important role in rib development (67).

Genes with higher expression in the cartilage sample

As expected, many of the genes that were most highly expressed in the C sample were either characteristic of or specific for cartilage – aggrecan, procollagens IX, X, XI, Sox9 and Indian hedgehog (68). The greater expression of BMP-7 (6.7× higher) in the C sample is consistent with several reports indicating its activity in promoting chondrogenic differentiation (69, 70). Similarly, cadherin 2 (N-cadherin), the most highly enriched (3×) cadherin in the C sample, is important for chondrogenesis (71).

Although both bone sialoprotein (4×) and osteopontin (5.3×) are important for bone formation (72, 73), osteopontin is also expressed by hypertrophic chondrocytes and deep layer articular chondrocytes (74). Both osteopontin and bone sialoprotein have been identified immunohistochemically in the matrix surrounding the hypertrophic chondrocytes of the MCC (57, 75–77), and MMP-13 has likewise been localized to

the deepest layer of hypertrophic chondrocytes in 1- to 10-day-old mouse MCC (78). Snail 1, enriched 3× in the C sample, is also highly expressed in hypertrophic chondrocytes (79), where it is thought to downregulate collagen II and aggregan synthesis, probably via fgfr3 signalling (80).

Conclusions: implications for orthopaedic therapies and regenerative medicine

While the results of this study must be considered preliminary, our findings offer new data regarding how prechondroblastic cells and their surrounding matrix differ in gene expression from the underlying chondrocytes of the mandibular condyle. Our study has confirmed the importance of the members of the FGF and TGF-β family of growth factors for proliferation and differentiation in the MCC, and provided potential insight into specific FGF ligands (e.g. FGF-13 and FGF-18) and other proteins (NCAM) that may be important for FGF signalling in the MCC. Moreover, the relative abundance of three Notch isoforms in the PC sample may be of importance in light of Notch's growing importance in regenerative medicine efforts (81). Secondly, our results provide information on the characteristics of the matrix of native MCC PC that may be of use in designing replacement tissues for the TMJ. But arguably the most important contribution of our results may derive from the identification of novel, unsuspected genes that are differentially expressed in the PC sample: the tooth-associated genes (tuftelin, tuftelin-interacting protein 11 and dentin sialophosphoprotein), VEGF-B and its receptors and associated cadherin, and Myf6 and its associated cadherin. Recent evidence has demonstrated that undifferentiated myogenic progenitor cells spontaneously express the osteoblastic-specific genes Runx2 and bone alkaline phosphatase (82). In addition, periosteal cells from adult humans can be made to differentiate into chondrocyte, osteoblast, adipocyte and skeletal myocyte lineages (83). Therefore, the relatively high expression of genes such as Myf6 and VEGF-B may indicate a degree of unsuspected plasticity in this bipotent cell population derived from an osteogenic lineage. Unfortunately, it is impossible to discern from our data whether certain of these genes are expressed by a sub-population of cells within the PC. However, our characterization of perichondrial gene expression may serve as a substrate for the burgeoning number of efforts attempting to regenerate the articular disc or MCC (84) or to upregulate growth at the MCC (85).

Clinical relevance

With the exception of some basic structural proteins, little is known of the genes that are highly expressed in the dividing cells of the MCC. Our study demonstrates differential gene expression in specific growth factor receptors and matrix proteins, as well as in novel, unsuspected genes that hint at an unrecognized plasticity of expression in these cells. Improved understanding of gene expression in native tissue will be essential for regenerative medicine efforts or attempts to upregulate the growth rate at the condylar cartilage for therapeutic purposes.

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