Biochimica et Biophysica Acta 1779 (2008) 330–340

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

A microarray approach for comparative expression profiling of the discrete maturation zones of mouse growth plate cartilage

Daniele Belluoccio, Bianca C. Bernardo, Lynn Rowley, John F. Bateman *

Murdoch Childrens Research Institute and the Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Parkville 3052, Victoria, Australia

ARTICLE INFO

Article history:
Received 12 November 2007
Received in revised form 19 January 2008
Accepted 20 February 2008
Available online 8 March 2008

Keywords:
Microarray
Microdissection
Growth plate cartilage
Endochondral bone
Gene expression

ABSTRACT

In vertebrates, longitudinal bone growth is the consequence of a complex series of events that take place in a specialized structure, the growth plate cartilage. Within the growth plate chondrocytes undergo a sequential maturation program from resting cells to proliferative, pre-hypertrophic, and ultimately hypertrophic end-stage chondrocytes. This process of chondrocyte maturation is under the control of the temporally and spatially regulated expression of a myriad of signaling molecules, transmembrane receptors, transcription factors, and structural extracellular matrix (ECM) proteins. One approach to the comprehensive definition of the key components of such complex interrelated pathways is the use of microarray expression profiling to catalogue transcriptome changes during chondrocyte maturation in the individual developmental zones of the mouse growth plate cartilage. However, this has not been achieved because of the difficulty in obtaining sufficient quantities of the individual growth plate cartilage zones to all microarray analysis. In this study we describe the development of microdissection methods for the isolation of tissue from the proliferative, pre-hypertrophic, and proliferative zone from one single mouse femur, RNA extraction and linear amplification of the RNA to allow interrogation of NIA 15k microarrays to generate comparative expression profiles. Verification of a subset of differentially expressed genes by RT-PCR and by *in situ* hybridization confirmed the reliability of this approach.

1. Introduction

Calcified skeletal elements are produced by either intramembranous or endochondral bone formation mechanisms [1]. The distinction between these rests on whether bone is formed from osteoblasts originating directly from aggregated mesenchymal cells (intramembranous), or whether ossification occurs after chondrocytes produce an intermediate cartilage model that serves as the scaffold for the bone formation process (endochondral). Intramembranous bone formation gives rise to the flat bones of the skull and face, mandible, and the lateral clavicles whereas the bones of the appendicular skeleton, vertebrae, and the medial clavicles are formed by an endochondral process.

In vertebrate long bones, the primary and secondary ossification centres are separated by a transverse cartilage region termed growth plate, which is responsible for longitudinal bone growth. The growth plate consists of chondrocytes and extracellular matrix and can be histomorphologically divided into various layers or zones reflecting the maturation states of the chondrocytes. These zones are referred to as the resting, the proliferative, the pre-hypertrophic, and the hypertrophic zone [2]. The resting chondrocytes appear round and are randomly distributed. The cells of this zone have been reported to have stem cell-like characteristics and represent a reservoir for cells that enter the cell cycle and become the chondrocytes of the proliferative zone [3,4]. The proliferative chondrocytes are of discoid shape and daughter cells align vertically to each other to form columnar structures in parallel orientation to the long bone axis. Eventually the cells enter a pre-hypertrophic stage where they are committed to reach their final maturation program, hypertrophy. After hypertrophy chondrocytes undergo apoptosis and matrix mineralization occurs in longitudinal septae between the chondrocytes [3].

The precise temporal and spatial coordination of chondrocyte proliferation and maturation, and the synthesis of regionally-specific extracellular matrix components, is critical for the organizational stratification of the growth plate cartilage and thus normal endochondral bone formation and growth. During growth plate proliferation and maturation several key regulatory pathways have been described. In particular, pathways involving Fgf, Bmp and Wnt families and their downstream effectors have been shown to act [5–8] along with the parathyroid-related peptide/Indian hedgehog (Pthrp/Ihh) negative feedback loop [9,10] as major controllers of cartilage maturation. The PTHrP receptor (Pthr1) is expressed in late proliferating and early hypertrophic chondrocytes [10,11] and acts to keep cells in a proliferating stage and to delay the maturation process to form pre-hypertrophic cells. Pre-hypertrophic and hypertrophic cells then synthesize Ihh [12,13] which by an unknown mechanism upregulates

* Corresponding author. Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville 3052, Victoria, Australia. Tel.: +61 3 83416422; fax: +61 3 83416429.
E-mail address: john.bateman@mcri.edu.au (J.F. Bateman).

1874-9399/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.bbagrm.2008.02.010

Pthrp synthesis and therefore increases the growth rate of proliferating cells. However, Ihh also directly stimulates chondrocyte proliferation via its receptor, patched (Ptc), which is most highly expressed in the proliferating zone [12]. FGFs have been shown to inhibit chondrocyte proliferation but promote terminal differentiation. Recently, it was demonstrated that Runx2, which have previously been shown to be expressed by hypertrophic chondrocytes, activates the *Ihh* promoter in vitro [14]. Conversely, FGF2 inhibits bone growth *in vitro* and induces a downregulation of *Ihh* and *Pthr1* gene expression [15].

While there is no doubt that the pathways that have been elucidated in studies so far are key elements in chondrocyte maturation in growth plate cartilage, it is highly likely that many important unknown regulatory components or unexplored members of signaling pathways and modifying components are yet to be revealed. One powerful approach for the detection of the spectrum regulatory components, and their downstream consequences, is the use of microarray expression profiling techniques to catalogue transcriptome changes during chondrocyte maturation in mouse growth plate cartilage. The use of the mouse as an experimental system is particularly advantageous because of the numerous genetic models of chondrodyplasias already available, and the relative ease of producing further transgenic models to test the functional consequences of ablation or overexpression of new members of hypothesized regulatory pathways. Comparative microarray expression profiling of the discrete zones of mouse growth plate cartilage maturation has not been achieved because of the difficulties in microdissection of sufficient quantities of the individually identifiable cartilage zones to obtain adequate amounts of mRNA for microarray interrogation. In this study we have validated microdissection and linear mRNA amplification methods that allowed us to conduct comparative microarray expression profiling on the proliferative, pre-hypertrophic and hypertrophic zones of mouse growth plate cartilage.

2. Materials and methods

2.1. Dissection and RNA preparation

14-day old (P14) Swiss white mice were sacrificed in accordance with Institutional Animal Ethics guidelines and femurs were dissected. The cortical bone of the diaphysis was removed while leaving the distal metaphyseal growth plate and the adjacent epiphysis intact. The tissue was immersed in Tissue-Tek OCT embedding compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in isopentane and stored at −80 °C. For later microdissection, 160 6 μm sagittal sections from one femoral growth plate were cut on a cryostat (Frigocut 2800, Reichert-Jung, Nussloch, Germany), mounted on RNase-free SuperFrost slides (Menzel, Braunschweig, Germany), fixed in 70% ethanol, washed in RNase-free water, and dehydrated in 70%, 95%, and 100% ethanol for 1 min each and air-dried. Slides were then immobilised on an inverted microscope (Leica, Wetzlar, Germany) and approximately 2000 cells from the proliferative (PR), pre-hypertrophic (PH), and hypertrophic (H) zones were dissected by using an ophthalmic scalpel (Feather, Osaka, Japan) that was immobilised on a xy-stage by a custom-made device. The tissue was then harvested into RNase- free eppendorf tubes and total RNA was extracted using the PicoPure RNA isolation kit (Arcturus Bioscience, Mountain View, CA) and contaminating genomic DNA was removed after on-column DNase digestion (RNase-free DNase set, Qiagen, Hilden, Germany). Total RNA from each zone was linearly amplified [16] in two rounds using the MessageAmp aRNA kit (Ambion, Austin, TX) following the manufacturer's instructions.

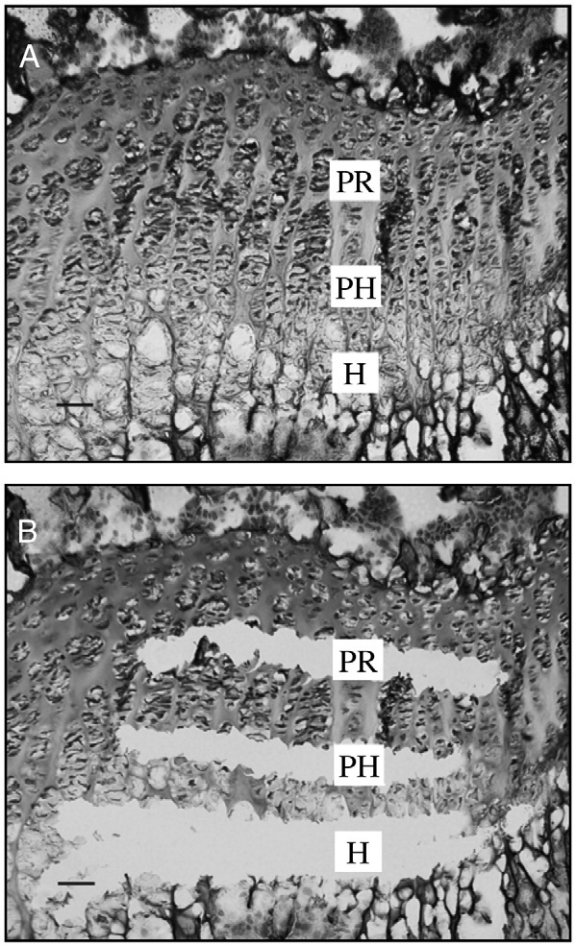

Fig. 1. Microdissection of chondrocytes from three different maturation stages. Sagittal cryosection of a distal femoral growth plate from a two week- old mouse before (A) and after microdissection (B) of proliferative (P), pre- hypertrophic (PH) and hypertrophic (H) zones. The section was stained with hematoxylin and eosin. Scale bars = 50 μm.

Table 1
Oligonucleotide primer sequences used for reverse transcriptase–polymerase chain reaction

Target (GenBank accession no.)	Primer	
	Forward	Reverse
Aggrecan (L07049)	ACCCCAACACCTACAAGCACA (bp 6452–6472)	AAAGCGACAAGAAGACACCA (bp 6604–6623)
ADAMTS-1 (BC050834)	CTGGGCAAGAAATCTGATGA (bp 4545–4564)	AAGCACAGCCACAGTTTATCA (bp 4815–4835)
Type X collagen (X67348)	TGTGTGCCTTTCAATCGAGTG (bp 7910–7930)	TCCGGGCTTTAATAAGTGAGG (bp 8039–8059)
Cyclin dependent kinase inhibitor 1c (AK077844)	ATCCCTCTGCCACGCAACT (bp 1561–1579)	AGGAACCATTTCGACTGTCTG (bp 1762–1782)
Insulin-like growth factor 2 (M36332)	TGTCCAGCAACCATCAGTGAA (bp 771–791)	TGGGTTGTTTAGAGCCAATCA (bp 1011–1031)
Alkaline phosphatase (X13409)	TTGCCAAGCTGGGAAGAA (bp 2175–2192)	GTTCCCTTTTAACCAACACCA (bp 2344–2364)
GAPDH (M32599)	GAATACGGCTACAGCAACAGG (bp 989–1009)	TTATTATGGGGGTCTGGGATG (bp 1143–1163)

2.2. cDNA microarray analysis

Amplified RNA (aRNA) from each zone was analysed on a Bioanalyzer 2100 using an RNA 6000 Pico LabChip kit (Agilent Technologies, Palo Alto, CA). 1.25 μg of aRNA originating from each growth plate zone was mixed with 250 ng of random hexamer primers (Roche Diagnostics, Basel, Switzerland), heated at 70 °C for 10 min, and snap-cooled on ice. The oligo/aRNA mix was then employed for complementary DNA (cDNA) synthesis at 42 °C for 2 h in a total reaction volume of 30 μl using 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.2 mM dTTP, 0.3 mM amino-allyl dUTP (aa-dUTP) (Amersham Biosciences, Uppsala, Sweden), 10 mM DTT, 1x first strand buffer, and 150 units of SuperScript II (Invitrogen, Carlsbad, CA). The RNA templates were hydrolyzed at 65 °C for 15 min after addition 10 μl of 1 M NaOH. The mixture was then neutralized with 25 μl 1 M HEPES buffer pH 7.0 and unincorporated aa-dUTP and free amines were removed by applying and spinning the mixture onto QIAquick purification columns (Qiagen, Hilden, Germany). The columns were washed and Cy3 or Cy5 monoreactive dyes (Amersham Biosciences, Uppsala, Sweden) were resuspended in 15 μl 0.1 $NaHCO_3$ pH 9.0 and pipetted directly onto the columns. The coupling reaction was left in the dark for one h at room temperature. The reactions were eluted with 80 μl of H_2O and then diluted with 400 μl of PB buffer. To remove uncoupled dyes the Cy3- and Cy5-labeled cDNA of one two-color hybridization experiment were loaded sequentially onto one fresh QIAquick column, washed, and eluted with 30 μl of H_2O. The labeled cDNA targets