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Comparative epigenomic analysis of bone tissue and primary osteoblasts

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Summary
Objectives: Epigenetic mechanisms, and in particular cytosine methylation in the promoter regions, modulate the expression of many genes. However, their role in skeletal homeostasis has scarcely been studied. In particular, it is not known if the patterns of methylation of bone cells in culture are a good reflection of that which occurrs in bone tissues. The aim of this work was to explore the possible differences in cytosine methylation in human bone and in osteoblasts.
Material and methods: To achieve this we carried out a genome-wide study, analysing the degree of methylation of 23,667 *loci* and comparing the results in samples of bone tissue and in cultures of primary osteoblasts.
Results: Overall, we observed a good correlation between the two sample types, both in the whole group of *loci* (r^2=0,87; $p<10^{-50}$), and in those located in genes involved in bone metabolism. However, some of the *loci* (7-8%) deviated from this general tendency and showed differences in methylation greater than 20%.
Conclusions: These results indicate that the methylation data obtained in cultures are not necessarily a true reflection of that which occurs in tissues, which means that care should be taken when extrapolating such results to an *in vivo* situation.

Key words: *DNA methylation, epigenetics, osteoblasts.*

Introduction

Some common skeletal diseases, such as osteoporosis or arthrosis, have a clear tendency to familial aggregation, which suggest that their hereditary component is significant[1]. In fact, in various studies it has been estimated that heredity explains up to 50-80% of the variability in bone mass[2,3]. However, the allelic variants identified in studies of candidate genes and genome-wide association studies (GWAS) explain barely a small proportion of this hereditary component[4-6]. Epigenetic mechanisms may contribute to the explanation of this phenomenon. These mechanisms permit the adaptation of the expression of genes to environmental conditions. This includes DNA methylation, posttranslational modifications of the histones, the non-coding RNA and the general structure of the chromatin[7-9].

In human DNA, most of the cytosines which are followed by a guanine are methylated. It is thought that this gives stability to the DNA. However, in the promoter regions of many genes there are zones rich in cytosines followed by guanine (called CpG islands) which may be methylated or not[10]. The degree of methylation of these islands is correlated with transcriptional activity: in general, the greater the methylation, the lesser the gene expression[11,12].

There are scarcely any studies of CpG island methylation in bone or in osteoblasts, especially in humans. Nor is it known whether or not the patterns of methylation in CpG islands in the osteoblasts are comparable with those observed in bone. Therefore, the objective of this work was to explore the methylation of cytosines throughout the whole of DNA in samples of human bone, and to compare those results with the patterns of methylation in primary osteoblasts in culture.

Material and methods

Bone and osteoblast cultures

Samples were taken of trabecular bone in the femoral head of women undergoing hip arthroplasty (fractures, arthrosis), using a serrated trocar. The cylinders were obtained from the central region of the head, avoiding the subchondral bone and the areas of fracture and osteotomy, as has previously been described[13]. After extensive washing in PBS the samples were frozen in liquid nitrogen or placed in plastic flasks in Dulbecco's medium supplemented with 10% bovine serum and antibiotics to obtain the osteoblasts from the explants[14].

Analysis of the methylation

After pulverising the bone fragments the DNA was isolated by a procedure previously published[12]. A similar procedure was used to extract the DNA from the confluent osteoblast cultures, from first or second passes[15]. To analyse the methylation, methylation arrays were used (Infinium Human Methylation 27 DNA bead-chip analysis, Illumina) which examined around 27,000 CpG loci located in the promoter regions of some 14,500 genes. The degree of methylation of each locus is expressed as a value of β, which varies between 0 and 1 and is proportional to the methylation (0-100%). The details of the method have been published previously[16].

Analysis of the results

The values of β were multiplied by 100 in order to estimate the percentage of methylation. The average values methylation observed in 15 bones from patients with fracture and in 15 from patients with arthrosis, and who were included in an earlier study[16], were calculated. The average age was 77 years. The results were compared with the average methylation observed in two osteoblast cultures (one from a bone with fracture and the other with arthrosis), which, to reduce sources of variability, were analysed together in the same arrays as the bone samples. To compare the methylation in the two types of sample correlation and linear regression tests were used. Bioinformatic databases and relevant literature were searched in order to identify the genes related to bone.

Results

A total of 23,667 loci were explored. As is shown in Figure 1, when all the CpG loci explored were analysed together a direct correlation was found between the levels of methylation in bone and in the osteoblasts (r^2=0.88; $p<10^{-50}$). Also, in general terms, the average methylation in both types of sample was similar (slope of the regression line b=1.009; intercept -4). However, there was a significant number of loci which deviated from this relationship (Figure 1). To analyse whether these deviations depended on genes not related to bone a limited sub-analysis was carried out of 658 loci located in 319 genes which were clearly related to skeletal homeostasis. The result was similar to that in the overall analysis (Figure 2). There was a general correlation between the levels of methylation in the two samples (r^2=0.87; $p<10^{-50}$), but a significant proportion of the genes deviated from the general relationship.

Restricting the analysis to the 319 bone genes (in which 658 loci were explored), the methylation in bone was slightly higher than in the culture (average difference 3.8%; $p=2.4 \times 10^{-15}$; Figure 3). Specifically, of the 658 loci, 117 (17.8%) showed differences greater than 10%. Of these, 61% were more methylated in the bone tissue than in the culture, while in 39% of the loci the methylation was greater in the cultures. In 45 loci the difference in percentage methylation was greater than 20 points, the excess methylation being equally distributed, in this case, between the bone tissue and the cultures. The genes in which these loci were situated are shown in Table 1.

Discussion

The analysis of the epigenome, and in particular the pattern of DNA methylation, is a subject of growing interest, given the role which it plays in determining the pattern of gene expression across the different stages of differentiation of the cell lines, as well as in their adaptation to changing environmental conditions. Its role in some disea-

ses also appears to be important, especially in neoplastic processes[17]. In fact different studies have related the changes in the methylation of the promoters with alterations in the expression of genes facilitating or inhibiting the development of tumours[18-20]. However, little is known about the role of patterns of methylation in non-tumorous diseases of the skeleton.

One of the factors which makes the analysis of the epigenome difficult is that, differently from the genome, the epigenome is specific to each tissue. This is logical, given that the patterns of gene expression need to be aligned with the specific functions of the tissue (in fact, with those of each type of cell). Hence, given difficulties in obtaining samples of the skeleton, there is little information on the epigenome of bone.

Our group has recently published an analysis of the pattern of methylation in bone tissue in patients with osteoporosis and with arthrosis[16]. In this study we have used these data to compare them with the patterns of methylation in primary osteoblasts in culture, with the aim of determining the extent to which they are similar. This analysis is important in exploring whether or not cells in culture are a good reflection of the pattern in tissue and, as a consequence, if the changes induced by various manipulations of the cultures may be relevant to tissue. In this whole genome study, in which we analysed some 23,000 loci, we confirmed that, in general, there is a good correlation between patterns of methylation in bone and in primary osteoblasts in culture. However, some genes clearly deviate from this pattern. The deviation does not follow a systematic pattern, and affects both genes which have been related to bone metabolism as well as others. Overall, 17-18% of the loci (located in genes related or not to bone metabolic pathways) had deviations in the degree of methylation of greater than 10%. The proportion of genes with differences higher than 20%, certainly significant from a biological point of view, was 7-8%, similar in the loci as a whole and in those located in the sub-group of genes related to bone. There are various reasons which may explain these differences. On the one hand, the culture itself may induce phenotypical changes in the cells, including changes in the patterns of expression and gene methylation. On the other, in bone tissue there are various cell lines, not only osteoblasts, which are not represented in the cultures. Unfortunately, it is not possible to cultivate osteocytes, a type which is highly abundant in bone, to carry out a comparative study similar to that carried out with osteoblasts.

In conclusion, the results of our study indicate that there is a good overall correlation in patterns of methylation between bone tissue and osteoblasts. However, some genes have clearly divergent patterns, with a similar frequency in the subgroup of genes related to bone metabolism to that in the genes analysed in general. Therefore, methylation data observed in culture may not be representative of the situation *in vivo*.

Figure 1. Percentages of methylation in bone tissue and in osteoblast culture across all loci analysed

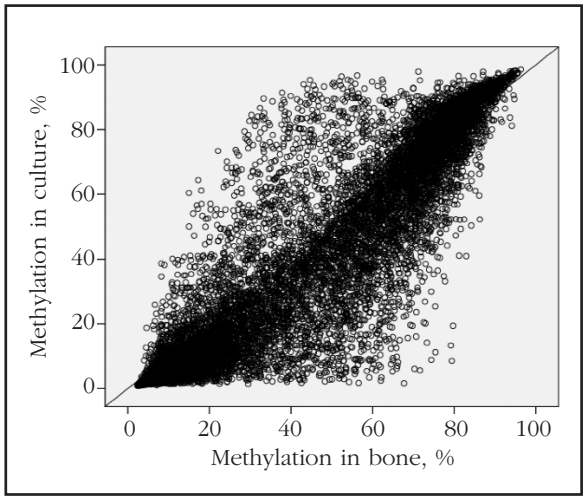

Figure 2. Percentages of methylation in bone tissue and in osteoblasts cultures in loci corresponding to genes related with bone metabolism

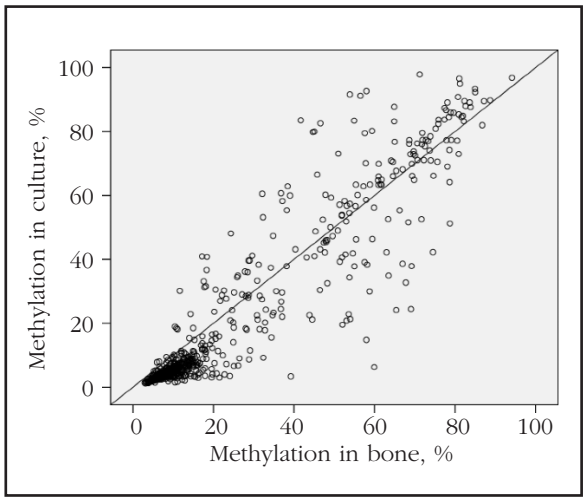

Figure 3. Distribution of frequencies of the differences in methylation between bone tissue and osteoblasts in culture. Only the data corresponding to the genes relating to bone metabolism are shown

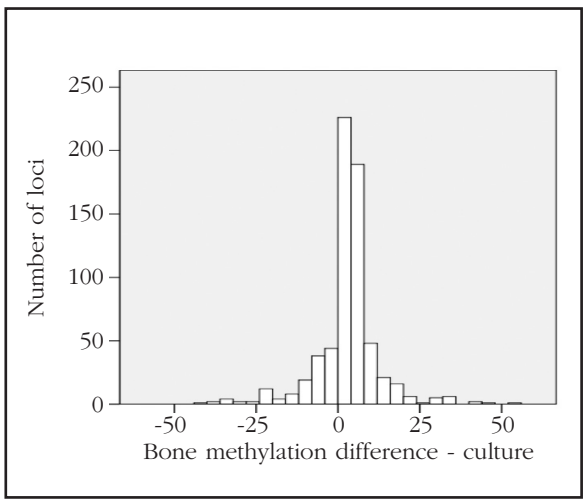

Table 1. Genes related to bone metabolism in which were observed differences in methylation between bone tissue and primary osteoblast cultures greater than 20 percentage points. The number of CpG loci with differences in methylation is indicated

Gen	Nº loci
ACVRL1	1
AMH	1
APC	1
AR	2
ATP6V0D2	1
BGN	1
CDKN2B	4
CHRD	1
COL3A1	1
CXCL12	1
DLX5	1
ENG	1
FGF1	1
FGFR1	2
FKBP1B	1
GDF5	1
IL1B	1
IL1RN	1
ITGAM	1
LGALS1	1
MAP4K1	1
MAPK1	1
MAPK10	1
MSX1	7
NR3C1	1
PTHLH	1
PTHR1	1
SFRP1	1
SMAD2	1
TGFB3	1
TNF	1
TRAF1	1
WISP1	1
WNT6	1

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