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# Response of osteoblasts to compounds of strontium or calcium: proliferation, differentiation, mineralisation and whole genome response

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## Summary

*Background:* The mechanisms which trigger osteogenesis are not yet clear. The objective of this study was to evaluate the role of strontium and calcium, provided in different molecular forms, as inductors of different mechanisms of osteoblast stimulus, including proliferation, differentiation and mineralisation of preosteoblast cells. The whole genomic response was also investigated using the microarray technique.
*Methods:* An experimental study was designed with murine preosteoblast cells MC3T3-E1, which were stimulated for 3 hours and 7 days. Biochemical and genome gene expression studies of mouse (Affymetrix) were carried out.
*Results:* Strontium bonded with ranelate (SrRn) was the most powerful inductor of the capacity of mineralisation in comparison with the other compounds used (2.55 times that of the control). The studies of whole gene expression showed that after 3 hours 2030 genes change, of which 1644 are specific to this phase. On the other hand, after 7 days of treatment only 329 genes change, of which 147 are specific. The biological processes most enriched after 3 hours are those involved in the regulation of transcription (147 genes), metabolic processes (140 genes) and protein phosphorylation (44 genes) among others, while at 7 days these are changes relating to the cell cycle (18 genes) and carbohydrate metabolism in general (12 genes).
*Conclusion:* Strontium bonded with the ranelate anion performed as the most powerful inductor of osteogenesis compared with other anions such as chloride or the hydroxides. The stimulation for 3 hours showed greater changes in gene expression in comparison with 7 days. The biological processes affected may be useful in speculating on the signalling cascades involved in the activation of the osteoblast, and on new molecular targets for therapeutic purposes.

**Key words:** *osteoporosis, osteogenesis, strontium, calcium, gene microarray, gene expression.*

## Introduction

The skeleton provides support and mineral equilibrium to the organism. Skeletal tissue is formed during growth and is maintained through adult life by the continuous renewal of the bone matrix, through a process known as bone remodelling. In this process an important role is played by two types of cell, osteoclasts and osteoblasts. During growth bone formation exceeds resorption, resulting in a net gain. Conversely, during aging a disequilibrium occurs which results in a negative bone balance[1]. Extrinsic mechanisms, such as changes in levels of hormones and growth factors, and intrinsic mechanisms associated with cell senescence, may cause the dysfunction of the osteoblasts[2,3]. This causes an increase in the number of bone remodelling units, which encourages trabecular perforation and a reduction in endocortical bone which in turn cause reduced bone resistance[4,5].

With the aim of limiting the excess resorption which follows the menopause, a series of antiresorptive drugs has been developed, such as bisphosphonates, monoclonal antibodies against cytokines involved in osteoclastic differentiation, cathepsin K inhibitors, etc[6,7]. Their long-term effects are, however, not known[7]. A significant challenge in the treatment of osteoporosis is the identification of strategies capable of reversing the deterioration of bone formation linked to age. To date, the number of anabolic agents which promote osteoblastogenesis is limited. The availability of parathyroid hormone (PTH) was a significant advance in the treatment of osteoporosis[8]. The intermittent provision of PTH increases bone formation in patients with osteoporosis, which results in an increase in trabecular bone mass and cortical thickness[8-10]. However, this anabolic treatment has some limitations linked to the low half-life and high cost of this molecule.

Strontium (Sr), a cation close to calcium in the periodic table has been shown to act pharmacologically on bone metabolism[11,12]. In certain experimental models it appears to develop an anabolic action, which has generated interest in associated pathways capable of promoting bone formation. In ovariectomised rats (OVX), an animal model for postmenopausal osteoporosis, it was observed that, both short- and long-term, treatment with Sr impedes the loss of trabecular bone induced by deficiency in estrogens[13,14]. Controlled clinical trials in postmenopausal osteoporotic women showed that treatment with Sr reduced the relative risk of vertebral fracture in comparison with the placebo group[15], as well as the risk of all non-vertebral fractures and hip fracture, as was analysed in a subgroup of patients of advanced age[16,17].

Various mechanisms may explain the reduction in risk of fracture induced by Sr in osteoporosis. One of the possible mechanisms is the increase in bone mineral density (BMD)[18]. Another would involve the effect of Sr on bone resorption and formation[19]. In support of this finding, it was found that 12 months of treatment with Sr causes an increase in the number of osteoblasts, formation of the matrix and a reduction in the number of osteoclasts in patients with osteoporosis[20,21]. In addition to the effects on bone cells and bone microarchitecture, Sr can increase bone resistance by means of changes in the properties of the bone matrix. A small fraction (less than 10%) of strontium is incorporated in the bone[22]. Given this, the beneficial effect of Sr on bone resistance cannot solely result from its pharmacological effects on the bone cells, bone remodelling and the microarchitecture, but may also arise from its effects on the properties of the bone matrix. Therefore, more basic and clinical analyses are required to clarify these effects at a molecular level.

In this work, we have studied the effects of different compounds of Sr and a calcium salt on different parameters related to osteogenesis using preosteoblast cultures as a model. In addition, we carried out a whole genome study by means of microarray with the aim of obtaining information on the genes and signalling pathways involved in osteoformation. For this, we used a microarray of mouse GeneChip Mouse Gene 1.0 ST, which contains more than 750,000 probes which represent 28,000 genes. The genes were analysed at different times, short (3 hours, acute phase) or long (7 days, chronic phase). The changes in gene expression were grouped according to the most notable metabolic processes and those related to the proliferation, differentiation and activity of the osteoblasts.

## Materials and methods

*Cell cultures*

The murine preosteoblast cell line MC3T3-E1 (Sigma-Aldrich, St. Louis, US) was used. The cells were kept in a culture medium composed of α-MEM (Invitrogen, California, US) supplemented with 10% foetal bovine serum (FBS, Invitrogen, California, US), 2 mM L-glutamine, 100 u/ml penicillin and 100 µg streptomycin (Invitrogen, California, US) in an incubation cabinet at 37 ° C in a humid atmosphere with 5% $CO_2$. In order to carry out the tests they were left to grow until 60-70% confluence.

Before any stimulation the cells were kept for 24 hours in a medium with 2.5% FBS. After this period the cells were treated with strontium ranelate (SrRn, AK Scientific Inc., US), strontium chloride ($SrCl_2$), calcium chloride ($CaCl_2$) and strontium hydroxide ( $[Sr(OH)_2]$ (Sigma-Aldrich) at a concentration of 2 mM for the periods specified in each experiment . The treatments were carried out in a medium of α-MEM supplemented with 2.5% FBS, 2 mM L-glutamine, 100 u/ml penicillin and 100 µg/ml streptomycin. For the prolonged treatments, the cultivation medium was refreshed by 50% and the corresponding treatment added every 3 days.

*Cell proliferation test (XTT)*

The cells were seeded in a plate of 96 wells (density $4x10^3$ cells/well). They were incubated for 48

hours with the aforementioned stimuli. The cell proliferation was measured with the Cell Proliferation ELISA II test (XTT-assay, Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions. After the incubation period, a measurement of absorbance at 450 and 650 nm at 37° was taken in a Victor™X3 2030 Multilabel Reader (Perkin Elmer, Massachusetts, US.) plate reader.

*Measurement of alkaline phosphatase (ALP) activity*
The MC3T3-E1 cells were seeded in plates of 96 wells at a confluence of 60% and subjected to the aforementioned stimuli for 24 hours, 3 days and 7 days. After the treatments the cells were washed with PBS, harvested with Triton 100 0.1% PBS and sonicated at 4°C. After centrifuging at 4°C at 20,000 g for 5 minutes, the supernatants were harvested, from which the alkaline phosphatase activity was determined using the ALP Reagent test (Thermo scientific, Massachusetts, U.S.). The absorbance at 405 and 660 nm (reference absorbance) was determined at 37°C each minute for 10 minutes in a Victor™X3 2030 Multilabel Reader (Perkin Elmer, U.S.) plate reader.

The concentration of protein was determined using the Bradford colorimetric method (BioRad, Hemel Hempstead, United Kingdom). The ALP activity was expressed as µg of product formed per minute of reaction over the quantity of protein present in the supernatant (U/mg).

*Mineralisation tests*
To quantify the mineralisation alizarin red was used (ARS, Sigma-Aldritch, St. Lois, Missouri, U.S.) an organic colourant capable of bonding with the deposits of calcium in the cells. It is used therefore as a marker for the capacity to form a calcified matrix. For the experiments, the MC3T3-E1 cells were seeded in plates and incubated with the different treatments SrRn, $SrCl_2$, $CaCL_2$ and $Sr(OH)_2$ for 24 hours , 3 days and 7 days. Then the cells were washed with PBS and fixed with formaldehyde 10% for 15 min. After fixing, they were treated with a solution of ARS (40 mM, pH4.2) for 20 min at room temperature. To detect their capacity for mineralisation the cells marked with ARS were incubated with acetic acid 10% for 30 min at room temperature. Subsequently, the cell suspension was harvested and incubated at 85°C for 10 min followed by 5 mins on ice, to harvest the supernatants after centrifuging at 20,000 g for 15 min. Potassium hydroxide (KOH) 10% was added to each supernatant, and finally, the intensity of fixing with ARS was determined by measuring absorbance at 405 nm in a plate reader Victor™X3 2030 Multilabel Reader (Perkin Elmer, U.S.).

*Extraction of total RNA*
The cells were cultivated in triplicate at a concentration of 106 cells per sample. After this, they were divided into an equal number of samples with or without treatment with 2 mM of SrRn for 3 hours and for 7 days. After these periods of incubation the culture medium was eliminated and the extraction of total RNA was carried using the trizol method according to the maker's (Invitrogen, California, U.S.) specifications. The quantity and purity obtained was confirmed by determining absorbance at 260 and 280 nm using Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, Delaware, U.S.).

*Microarrays of gene expression*
The analysis of the expression of whole genome was performed using the GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, California, U.S.) microarray which has more than 750,000 probes, with a longitude of 25 oligonucleotides which represent 28,000 mouse genes. Biological triplicates were obtained for each condition: controls, 3 hours and 7 days of treatment. We started from 300 ng of total RNA, having previously analysed its integrity ((Bioanalizador, 2100 Agilent), in order to synthesise the single-stranded cDNA. Its later fragmentation and marking was carried out following the manufacturer's (Affymetrix, Santa Clara, California, U.S.) indications. The hybridisation took place over 17 hours at 45°C with rotation at 60 rpm in the oven recommended by Affymetrix: "GeneChip Hybridization Oven 640" (Affymetrix, Santa Clara, California, U.S.). After this period, the microarrays were passed through a wash process and, finally, the marking was carried out with streptavidin-phycoerythrin using the "GeneChip Fluidics Station 450" (Affymetrix, Santa Clara, California, U.S.). Subsequently, the microarrays were scanned with "GeneChip Scanner 3000 7G" (Affymetrix, Santa Clara, California, U.S.) and images of each of the samples obtained. The quality controls of these images were performed using the programme "Affymetrix Genechip Command Console".

*Statistical analysis of the results*
The normalised .CEL files of the images of the samples from all the experiments were analysed with the software Partek Genomics Suite version 6.6 (Partek Inc., St. Louis, Missouri, U.S.). The microarrays were normalised using the RMA algorithm. The differential gene expression was identified using ANOVA by means of a highly restrictive analysis using False Discovery Rate $<0.05$ (FDR$<0.05$).

The expression of the baseline genes which changed over time between the control at 3 hours compared with the control at 7 days were discounted from the analysis to enable the more specific observation of those genes which changed after the stimulation with SrRn.

The cell processes included the significant genes, both for the acute and the chronic phases, were analysed using the software Pathway Studio version 9.0, database ResNet9.0;2012Q3 (Mammal) (Ariadne Genomics, Rockville, Maryland, U.S.).

The biochemical and cell proliferation data obtained were analysed with the programme GraphPad Prism version 4 (GraphPad Software,

Inc., California U.S.). The comparative statistical study was carried out using single factor analysis of variance (ANOVA) and the Bonferroni test for the multiple comparatives. The level of statistical significance was established at values $p<0.05$, for all variables analysed.

## Results

*The effect of different salts of Sr in MC3T3-E1 pre-osteoblast cultures*

*Cell proliferation*

Figure 1A presents the effect of different salts studied on cell proliferation determined by the XTT method. All the stimuli induced cell proliferation negative with respect to the control after 48 hours of treatment. $CaCl_2$ induced the highest degree of proliferation, with an increase of 2.13 times, which was highly significant ($p<0.001$). All the salts of strontium produced a slight increase in cellular proliferation ($p<0.05$) in the period studied with respect to the controls. There were no significant differences between the salts studied.

*Cell differentiation (alkaline phosphatase and mineralisation)*

ALP is a marker for early osteoblast differentiation and an increase in its activity is considered to be an indicator of an increase in the activity of the mature osteoblasts. Figure 1B shows the activity of ALP with the different salts over 7 days. Measurements were also taken at shorter time periods (24 hours and 3 days) but no increase in ALP activity was observed with any of the stimuli (data not shown), therefore, during this period of time the cells still remained undifferentiated. However, the evaluation at 7 days produced an increase in ALP activity, such that the strontium as a chloride or hydroxide salt achieved an increase of 4.45 which was highly statistically significant ($p<0.001$). The salts $CaCL_2$ or SrRn showed a lower increase in the activity of ALP, of approximately 2 times with respect to the control, of statistical significance ($p<0.01$).

Since it is known that Sr is incorporated into the bone matrix, the impact of the salts of Sr and of $CaCl_2$ on the capacity of the mineralisation of the pre-osteoblasts MC3T3-E1 was evaluated. Similar to what was observed in the ALP tests no mineralisation was observed at 24 hours or 3 days (data not shown), but the test was positive at 7 days after the stimulus (Figure 1C). The SrRn achieved an increase of 2.55 times in mineralisation measured by ARS with respect to the control and 2.2 times if we compare it with other compounds of strontium ($p<0.05$), which also appear to induce mineralisation slightly when compared to the control. The cells stimulated with SrRn showed a greater capacity for mineralisation even than the $CaCL_2$ salt studied, with a strong statistical significance ($p=0.06$).

In conclusion, the Sr bonded with ranelate induced slight cell proliferation, and an increase in ALP activity sufficient to achieve the maximum mineralising capacity of all the salts investigated. With this knowledge, the investigation of the gene expression mediated by this salt was begun.

*Analysis of gene expression using microarray*

The effect of SrRn on the gene profile of MC3T3-E1 cells was also investigated over 3 and 7 days of stimulus. These periods of study were selected in earlier studies by our group, where we detected the most intense changes in gene expression related to signalling cascades Wnt and NFAT, both important routes in the process of osteogenesis[23]. The results obtained by microarray were made in triplicate and are represented in a principal components analysis (PCA) map (Figure 2). It is observed that the samples from the same condition resemble each other and therefore the ellipses are small. The fact that the ellipses do not overlap confirms to us that there are genetic differences with the other conditions. The samples included within the 3 hour stimulus were very different, especially in comparison with those not treated (control). After 7 days, the differences narrowed, which suggests that the impact on gene expression reduces in relation to the acute phase.

A comparative statistical analysis was carried out using single factor analysis of variance (ANOVA) with an FDR $\leq 0.05$. We observed that in the acute phase there are 2,030 genes which modify their expression, of which specific to this phase are 1,644 genes which change significantly when the samples are treated with SrRn. On the other hand, after 7 days of stimulus, 329 genes changes in a statistically significant way, with only 147 of those specific to the chronic phase. The remaining genes were common to the two phases. In this study those genes which changed their expression among the controls at 3 hours and 7 days were discounted and we focussed on the genes which changed specifically in each period of time. Represented in Figures 3A and 3B are the biological processes in which those genes with differential expression may be integrated. The representation is made according to the number of genes which change, from higher to lower, although it should be underlined that all the changes presented are statistically significant. The cellular processes differed significantly according to the period of treatment, 3 hours or 7 days, with SrRn. In the acute phase the cellular processes associated with those genes whose expression changed corresponded to the regulation of transcription (147 genes), to general metabolic processes (140 genes), to transport (89 genes, of which 52 are related to transport of proteins) and 44 genes related to the phosphorylation of proteins. On the other hand, processes such as cell death, DNA repair or response to DNA damage were less enhanced. In contrast, at 7 days of stimulation, chronic phase (Figure 3B), the changes were different and the biological processes most enhanced were those metabolic processes which are involved above all in the metabolism of glucose (6 genes) and of carbohydrates in general (6 genes), cell cycle and division (10 genes), cell prolifera-

tion (6 genes) and, to a lesser extent, those related to hypoxia and lipopolysaccharide metabolism.

## Discussion

In this work, we studied the effect of three compounds of strontium on murine MC3T3-E1 pre-osteoblasts. We observed that Sr promotes cell proliferation and differentiation, as well as mineralisation, but its potential changes according to the anion to which it is bonded. If in the culture medium there is a source of organic phosphate, we observe discrete zones of mineral deposits which contain hydroxyapatite. Among the strontium compounds studied, the Sr bonded with ranelate was the most powerful inductor of mineralisation in comparison with chloride or hydroxide (Figure 1C). This process was accompanied by a small increase in the activity of ALP associated with the osteoblast phenotype. Conversely, the highest alkaline phosphatase activity was observed with Sr bonded with both chloride or hydroxide, demonstrating the versatility of the action of the cation according to the molecular framework which accompanies it. The cells used in this study have been very well described and are therefore an excellent model for studies of mineralisation, however they have a low expression of ALP. Other subclones of the same cell line have been characterised which, in contrast, have high ALP activity and do not have mineralisation capability[24]. It is known that little ALP (0.05 U/mg) activity is required in order for inorganic phosphorus to be obtained *in vitro*[25]. In mesenchymal cells from bone medulla low ALP activity has also been observed, as well as their being capable of mineralisation[26]. Therefore, the activity of ALP in the samples stimulated with SrRn is necessary and sufficient to produce a mineralisation matrix.

The sequence of the induction of the formation of mineralised foci progressed in a regular order over the time periods studied of 0-1-3-7 days. Experiments carried out with ascorbic acid have shown that this process is prolonged to 2 to 3 weeks[27], showing the efficacy of the action of SrRn. Presumably, successive waves of change in gene expression occur over the period of 0-7 days required for the differentiation of the pre-osteoblasts to a mineralisation phenotype. We carried out studies of up to 21 days of treatment with the stimuli, observing that the most intense changes in the cell, such as gene expression and activation of signalling cascades related to osteoformation processes, occur very shortly after the stimulus[28].

Given the high degree of mineralisation observed, and the fact that this process can only be executed by mature osteoblasts, strontium bonded with ranelate (SrRn) is for

Figure 1. The effect of different compounds of Sr in MC3T3-E pre-osteoblast cultures. Cell cultures without stimulation and treated with $CaCL_2$ were used as controls. (A) cell proliferation during 48 hours of stimulation; (B and C) alkaline phosphatase activity; ALP and the capability of mineralisation determined by the alizarin red method after 7 days of treatment, respectively. The results are represented as the mean ± standard deviation of triplicates, *p<0.05, **p<0.01, ***p<0.001, comparisons made with respect to the control (0)

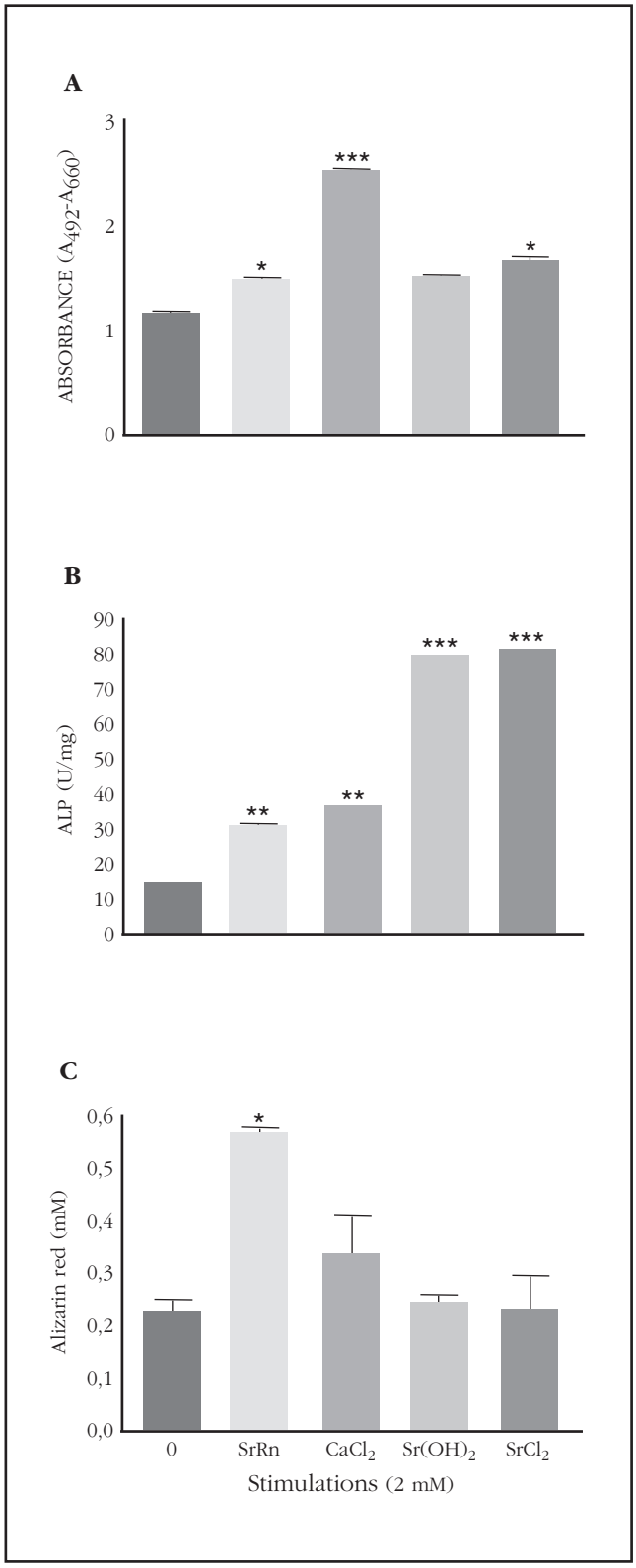
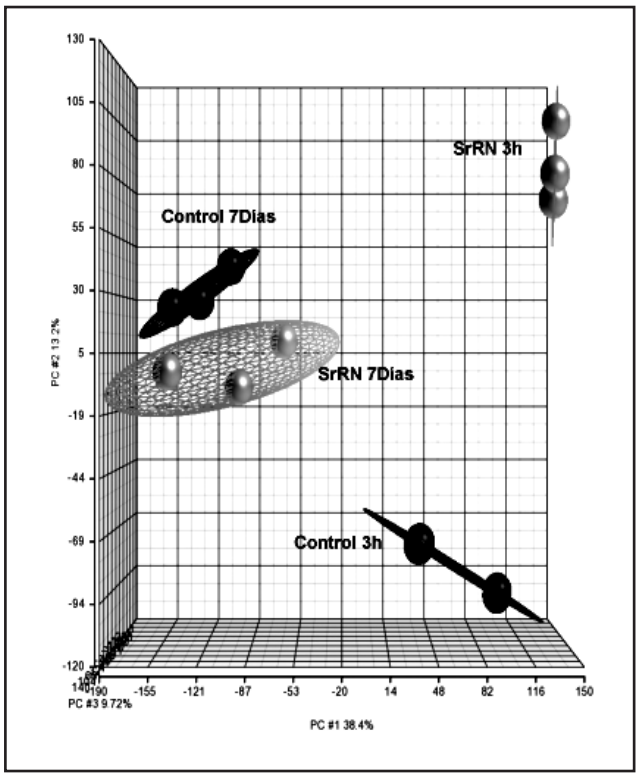


Figure 2. Representation of principal components analysis (PCA) map. All the samples are represented in 3D through a non-supervised analysis of principal components. The ellipses are drawn to include 61.3% of the genes in each group




us an excellent stimulus for the study of the cell processes which occur when osteogenesis or bone formation take place in these cells. We have used an expression matrix from the mouse which contains 28,000 genes. Earlier studies only used 588 or 8,700 probes[29,30]. Our matrix allows the investigation of the genes of numerous growth factors, cytokines, interleukins and their receptors, as well as key genes involved in different stages of embryo development. MC3T3-E1 cells are a well-established osteogenic clonal cell line, which provide an excellent model for the study of patterns of gene expression in osteoblast differentiation. In our study, the SrRn induced in these cells a process of maturation which can be divided into two phases, which result in the formation of a mineralised matrix (Figure 1C). Each of these phases requires strictly regulated expression of genes and transcription factors.

The first phase of this process of maturation (3 hours after stimulation, acute phase) is triggered on contact with the stimulus, SrRn. During this phase the MC3T3-E1 cells are not yet differentiated, and are not capable of producing a mineralisation matrix. However, in these cells the expression of many genes is activated, up to a total of 1,644, in which the 10 most enriched cell processes from our global gene expression study are shown in Figure 3A. In this stage many genes change which are related to the regulation of transcription, metabolic processes in general, molecular transport, etc. It is notable that among those general metabolic process enriched in this short period of time is protein phosphorylation-dephosphorylation, a process which participates in a large number of cell routes of great interest. Some of these have been shown to be involved in the maturation of the osteoblasts, such as the Wnt and NFAT signalling routes[31,32]. However, we currently have insufficient information regarding the cell pathways which are activated during the differentiation, proliferation and maturation of osteoblasts, in which doubtless the processes of protein phosphorylation-dephosphorylation are crucial.

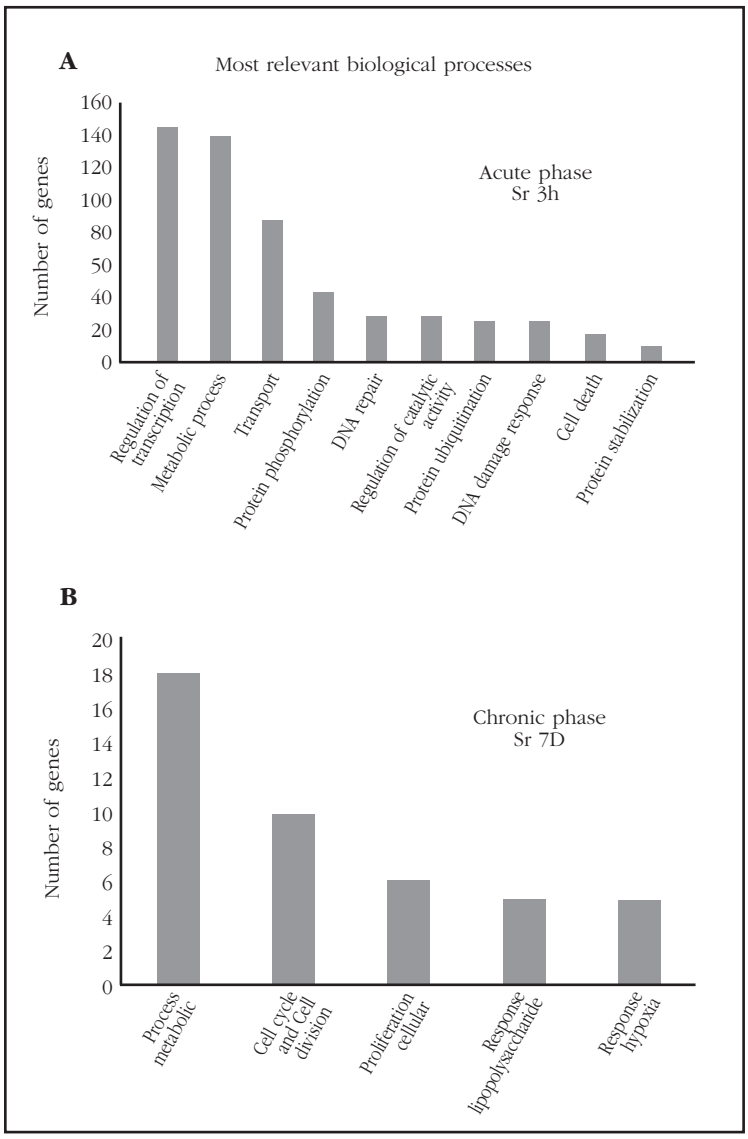
In accord with this pattern, in an earlier work we confirmed that the Wnt/β-catenin and NFAT pathways, both powerful osteoprogenitors, are activated within only 15 minutes of stimulus with SrRn, which rapidly induces changes in gene expression. In contrast, 21 day cultures do not show activation of these pathways (data not shown).

Once confluence is achieved, and coexistent with an increase in ALP activity and mineral matrix deposit (Figures 1B and 1C), the cells enter what is known as the differentiation phase (7 days). This is characterised by an increase in the formation of the bone matrix, and is associated with the change in expression of 147 genes. The cell processes which are increased in this phase are those related to the energetic state of the cells: carbohydrate metabolism in general, metabolic process of glucose and other carbohydrates, response to lipopolysaccharides or gluconeogenesis. This makes this cell line an excellent model for the study of the molecular events involved in the process of osteogenesis. The microarrays allow the simultaneous monitoring of a great number of genes associated with the metabolism of bone, which enables the detection of potential targets for therapeutic or diagnostic purposes. The use of microarrays is becoming ever more accessible and the data generated from this type of approach are largely descriptive. However, the general information which is obtained is a very useful baseline for more analytical studies of functional routes which come into play during cell differentiation, an event which is mediated by receptors, transcription factors, proteins, enzymes, etc., which are in turn regulated by changes in gene expression.

The analysis here presented offers a dynamic vision of these events during the differentiation of osteoblasts. The results of the expression matrix which is presented here complement earlier studies[29,30]. However, many earlier gene studies in osteoblasts were analysed under different conditions, with different cell lines, or under induction by different agents. The ability to compare data collected from hundreds of genes under a combination of conditions with data from other systems strengthens our general understanding of the

molecular basis of osteogenesis. These data are valuable not only for a better understanding of osteogenesis but also for comparison with other types of tissues. This information should help in the development of more efficacious treatment for bone disorders and in the prediction of secondary effects on bone metabolism of drugs which are aimed at the same factors for intervention in other diseases.

Figure 3. Representation of the most significant biological processes of each phase in which are integrated those genes modulated by the treatment. The representation is shown from higher to lower according to the number of genes which change. All are statistically significant (p<0.001)

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