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The Culture Medium Conditioned by Adipose Tissue Is Able to Regulate the lncRNA HOTAIR, HOXC11, HOXC12, Osx and SATB2 Gene Expression in MSCs during Osteoblasts' Differentiation

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Abstract

The osteogenesis is a complex process that involves an accurate control of bone development, growth as well as remodeling during postnatal life. In particular, osteoblasts arise from mesenchymal stem cells through a process regulated in several steps that leads to the osteogenic differentiation. Class I homeobox genes (HOX in human and Hox in mice), are 39 transcription factors, mainly involved in the regulation of embryonic development. It has been shown that HOXA10 was able to activate several osteoblast related genes like Osx. The HOXA10 gene was considered a key factor able to control osteogenesis. In the last decade, several non-coding RNAs (ncRNAs), have been identified like crucial factors during osteogenesis. HOTAIR is an lncRNA related to Osteosarcoma and able to induce the Epithelial Mesenchymal Transition. The aim of this paper was understood the role of HOTAIR and HOXC locus in the control of Osteoblasts development in MSCs treated with adipose tissue obtained with Lipogems method.

Keywords: Osteoblasts, HOX genes, HOXC, HOTAIR, Osteogenesis, Memory program

Introduction

Bone tissue is a dynamic structure capable of supporting mechanical variations, due to different environmental conditions. Moreover, the old tissue can be replaced with new matrix using the high potential of bone remodeling. Specific cells, like Osteoclasts and Osteoblasts, lead the resorption process and deposit of new bone matrix. The osteogenesis is a complex process that involves an accurate control of bone development, growth as well as remodeling during postnatal life. In particular, osteoblasts arise from the osteogenic differentiation of

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mesenchymal stem cells through a process regulated in different steps. Although, the understanding of the osteogenesis transcriptional control is increased considerably, the molecular regulatory basis is still poorly understood (Zhu., et al. 2013). In the near future, the increase of knowledge about the role of specific transcriptional factors in the control of Osteoblasts differentiation consequent to post-genomic era will be expected, in order to identify the molecular mechanisms useful in the tissue regeneration and tissue engineering methodologies to apply in clinical practice (Nair., et al. 2014). 500 genes regulate bone development; particularly homeobox-containing genes like Osterix (Osx), special AT-rich sequence binding protein-2 (SATB2), HOX-family are the best candidates in the control of bone tissue organization (Weiss., et al. 1995).

Class I homeobox genes (HOX in human and Hox in mice), are 39 transcription factors involved in the regulation of embryonic development program; The HOX genes are characterized by a sequence of 183 nucleotides encoding a hoemodomain of 61 amino acid that binds to DNA, as biological gripper, activating or repressing specific genes (Ferber., et al. 2000; Foucher., et al. 2002). Moreover, the HOX genes, conversely of others gene networks, are not spread along the genome, but are located on four chromosomes and are arranged in specific chromosomal clusters or loci (HOXA chr 7p15.3, HOXB chr 17q21.3, HOXC chr 12q13.3 and HOXD chr 2q31), each having from 9 to 11 genes. Based on the position within the locus and similarity of homeobox sequence, corresponding genes of the four clusters, can be aligned with each other in 13 paralogous groups (Figure 1) (Barnes., et al. 1995). The HOX network is active in adult human tissues and organs, controls the spatial-temporal generation of biological structure expected during embryonic development, regulates the cell memory program and control the cell phenotype. Epigenetic change in the memory program is critical in the onset and evolution of normal organs and tissue, but deregulation of genes related to cell memory program plays a key role in the change of cell phenotypes and in the determination of several pathologies (Bantignes and Cavalli 2006).

Three genes families control the cell memory program: Polycomb, able to block the interaction, DNA-chromatin leading to silencing of HOX genes. Trithorax genes are able to induce the mRNA transcription through an open configuration of DNA-chromatin interaction, and lead the activation of HOX genes. Finally, the HOX family is involved in the control of the cells phenotype, through a specific gene program mainly by means of an accurate regulation of the mRNAs transcription (Figure 2) (Ghering and Hiromi 1986, Procino A. 2014).

Many evidences have shown the involvement of Homeobox genes in bone formation: the upregulation of HOXA2 is crucial for repress osteogenesis. (Dobreva, et al. 2006). Recently, It has been studied the role of HOXA10 in the control of osteoblasts differentiation; the hyper-expression of HOXA10, was able to activate several osteoblast related genes like Osx; this transcription factor controls bone mineralization and osteoblasts differentiation. Therefore, HOXA10 gene was considered as a key factor for: i) the proper timing expression of specific osteogenesis markers; ii) correct mineral and matrix deposition during osteoblasts maturation (Gordon, et al. 2011)

It has already demonstrated that Osx plays a crucial role during bone formation. Osterix is able to manage the expression of specific genes related to osteogenesis. His zinc-finger domain is crucial during the transition from pre-osteoblasts to functional osteoblasts (Baek, et al. 2009; Nakashima., et al. 2002).

Osteogenesis is a process controlled by several transcriptional factors, one of the most important titled special AT-rich sequence binding protein-2 (SATB2); SATB2 appears to exert different roles in craniofacial patterning, osteoblast differentiation and maturation, bone formation. SATB2 is able to activate or repress several osteoblasts related genes, controlling the osteoblast differentiation (Yamaguchi., et al. 1991; Wozney., et al. 1995; Zhao., et al. 2013).

Several non-coding RNAs (ncRNAs), have been identified like crucial factors during osteogenesis. In details, miR-29b, 22 miR-204/211 e miR-182, 24 are able to control bone development during Mesenchymal Stem Cells (MSCs) differentiation. Moreover, it has been demonstrated the ability of lncRNA MEG3, H19 and ANCR to regulate, in MSCs, the expression of different osteoblast related genes (Zhuang., et al. 2015; Zhu., et al. 2013; Wang., et al. 2016).

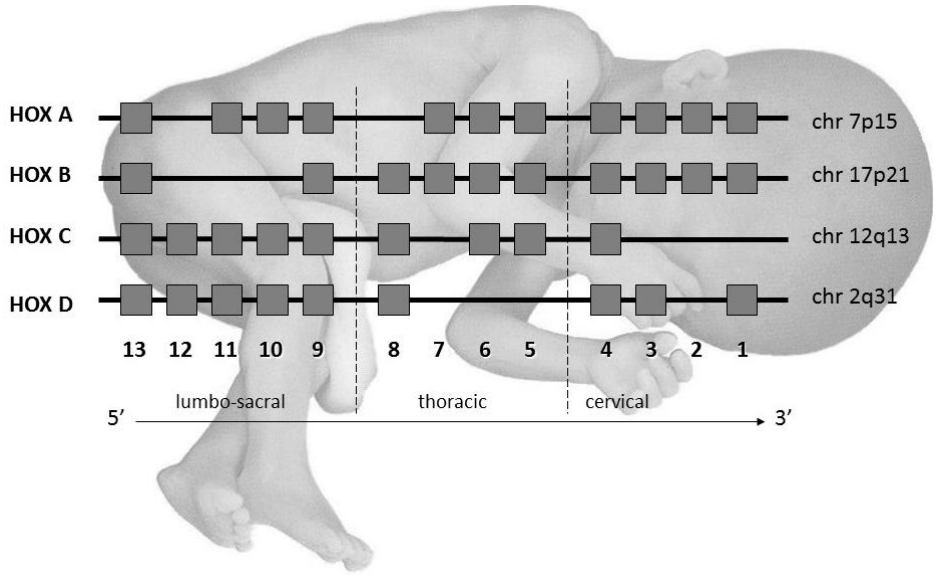

Figure 1. The HOX gene arranging during human embryonic development (see the text).

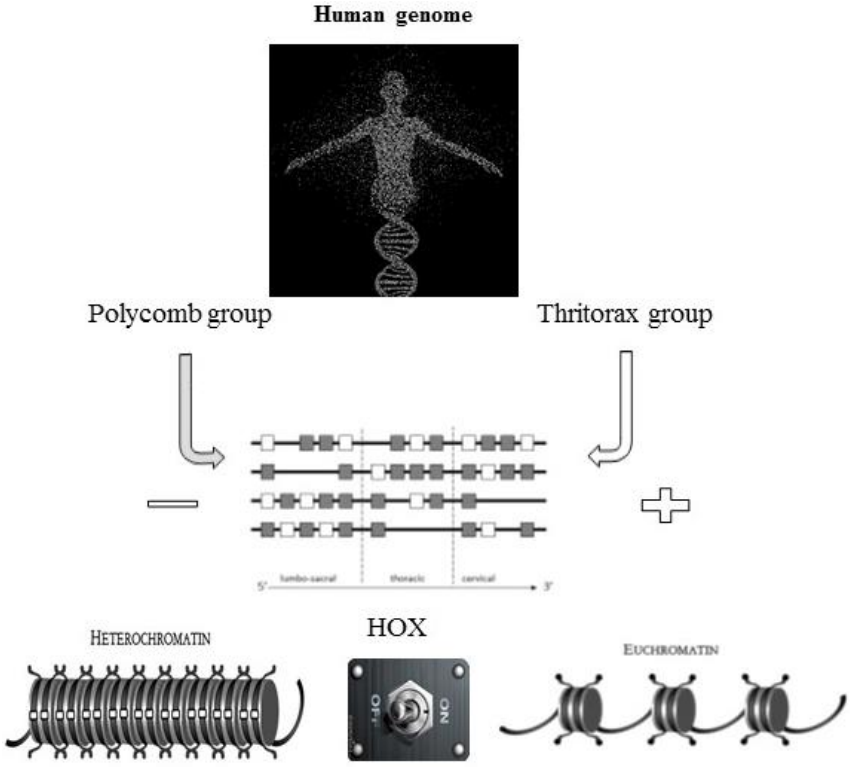

Figure 2. The HOX genes and Cell Memory Program control (see the text).

Recently, it has been identified 231 lncRNA able to interact with HOX genes; in particular, one of those titled lncRNA HOX antisense intergenic RNA (HOTAIR) was identified in 2007. HOTAIR is transcribed from HOXC locus located at chromosome 12q13.13, in intergenic and anti-sense position to the flanking HOXC11 and HOXC12 genes (Rinn, et al. 2007; Kogo., et al. 2011; Tano and Akimitsu 2012; He., et al. 2011). HOTAIR is the first lncRNA that control the gene expression with trans mechanism. In details, it is able to block the transcriptional control of the HOXD locus on the chromosome-2, including the

unique Polycomb Responsive Element (PRE) to date identified in the genome. Moreover, the lncRNA HOTAIR and HOX genes are considered key factors in the control of the epithelial cell memory program (Procino, 2016). The HOTAIR is involved in pathogenesis and progress of multiple tumors. Recently, HOTAIR has been identified in Osteosarcoma (OS) and its upregulation confirmed that HOTAIR play a crucial role in the control of bone tissue deregulation (Zhou, et al. 2016)

The aim of this research was to study the osteoblasts differentiation considering the potential involvement of lncRNA HOTAIR and two HOX gene family members (HOXC11 and HOXC12) in mesenchymal stem cells (MSCs) treated with adipose tissue, obtained with Lipogems method.

Materials and Methods

1. Processing of Adipose Tissue with the Lipogems Device

Human subcutaneous adipose tissue samples were obtained with patient informed consent from abdominal lipoaspiration procedures performed before arthroscopic rotator cuff repair and processed by using the Lipogems device, according to the manufacturer's instructions previously described. Avoiding the presence of air, the lipoaspirate tissue was subjected to a first cluster reduction, obtained by pushing the lipoaspirate from the syringe into the device through the first large filter (blue cap), and allowing the corresponding quantity of saline to exit towards the wasting bag. The five stainless steel beads contained in the device were essential to obtain a temporary emulsion between oil, blood, and saline, which could be washed away against density following the current of saline moved by gravity in the wasting bag. After this washing step (the following solution appears clear and the lipoaspirate yellow), the saline flux was stopped and the device was reversed (gray cap up), leading to the second adipose cluster reduction. Such reduction was obtained by pushing the floating adipose clusters through the second cutting hexagonal filter (grey cap), pushing fluid from below with a 60mL syringe. The reduced cluster was collected in another 60mL syringe placed above and positioned to gently decant the Lipogems product by gravity in order to remove the excessive saline solution. A mean of 50mL of lipoaspirate was collected and processed by the Lipogems device to obtain an amount of 20mL of the final Lipogems product, which was transferred to several 10mL syringes to be injected in the same patient or used in our experiments. (Randelli, et al. 2016).

2. Preparation of Conditioned Medium (CM) from Lipogems (LG).

LG was washed in PBS at least three time by centrifugation at 250 g for 10.' LG was then aspirated and seeded (around 2 ml) in T25 flask in5 ml of IMDM serum free medium (a similar amount of LG was used for cell extraction with collagenase). The flasks (five for each LG preparation) were incubated for 5 days at 37°C in 5% CO_2. At the end of the incubation, the medium was aspirated and centrifuged at 250 x g for 10' LG-CM was filtered 0.22 μ, aliquoted and stored at -20°C until use (Cesarani, et al. 2016).

3. Cells Culture and Culture Medium

Osteoblasts culture medium was prepared using DMEM-low glucose (Sigma-Aldrich), 10% FBS (BioWest), 4mM L-glutamine (Euroclone), and 1% antibiotic-antimycotic mixture (Euroclone), supplemented with 0.1 μM dexamethasone, 50 μg/mL L-ascorbic acid-2-phosphate, and 10mM β-glycerophosphate (all reagents from Sigma-Aldrich). Mesenchyml Stem Cells Medium was constituted with alpha-MEM without L-glutamine (BioWhittaker), FBS (BioWest), 1% antibiotic-antimycotic mixture (Euroclone) and 4mM L-glutamine (Euroclone). The MSCs (LGC Milan, Italy) were seeded in Multiweel™ 6 wells. We seeded 3 x 10^4 for each well. In order to study the induction of osteogenesis-related genes, we perform three different multiwell: i) MSCs treated with alpha-MEM medium; ii) MSCs treated with Osteoblast medium; iii) MSCs treated with Lipogems Conditioned Medium (LG-CM). The cell were stimulated for

20 days and then we refresh the medium each three days (Randelli, et al. 2016).

4. MTT assay

MTT, measuring the mitochondrial dehydrogenase activity. MSCs cells were seeded into 96-well flat-bottomed tissue culture plates respectively at 10^4 cells/well and treated as described in the paragraph 3. After 24 hours of incubation, the culture medium was replaced with 200μL/well of dilutions. Elapsed further 24 hours, the medium was substituted with 100μL/well of MTT solution (1 mg/ml) in PBS, and the cells were incubated for an additional hour at 37°C in a 5% CO_2 atmosphere. After the solution was removed, 100 μL/well of DMSO were added and the plates were swirled gently for 10 min. The optical density of each well was immediately measured in a spectrophotometer (Sunrise ™, Tecan, Mannederf/Zurich, Switzerland) at 590 nm. The optical density of cells cultured in the medium plus saline solution without the mini screw extracts was used as control for 100% of cell viability and as a reference for the determination of the cytotoxicity (%) in the assay. At least four independent experiments were performed in quadruplicate (Borelli, et al. 2017).

5. Alamar Blue Assay

MSCs (10_4 cells/well) were seeded in 96-multiwell plates. Cell proliferation of four replicate samples was assessed by alamarBlue assay (AB) at 7days and 14 days. AlamarBlue assay uses a visible blue fluorogen probe resazurin, which is reduced to a red fluorescent compound (resorufin) by cellular redox enzymes of the mitochondrial respiratory chain. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and proliferation [28]. The AB assay was performed according to the manufacturer's protocol (BioSource International, Camarillo, CA, USA). At predetermined culture intervals, 200 μL of alamar Blue dye were added directly into culture media at a final concentration of 10 % and the plates were returned to the incubator for 4 h. As a negative control, AB was added to the medium without cells. The percentage AB reduction was calculated from the values of optical density at 540 and 590 nm using the manufacturer's formula (Gandolfi M.G. et al. 2014).

6. RNA Extraction and Analysis

Total RNA was isolated from MSCs culture cells, using RNeasy Micro-Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Samples were treated with RNase-free DNase (Qiagen GmbH) to prevent amplification of genomic DNA. One microgram RNA was subjected to cDNA synthesis for 1 h at 37°C using the Ready to go You-Primer First-Strand Beads kit (Amersham Biosciences cod. 27-9264-01) in a reaction mixture containing 0,5 mg random hexamers (GeneAmp RNA PCR Random Hexamers Set N808-0127 Applied Biosystems, Foster City, CA).

7. Real Time-PCR (QRT-PCR)

QRT-PCR will be performed using Taq-Man or technology (QRT-PCR StepOne). This assay uses a specific oligonucleotide probe, annealing between the two primer sites, which is labelled with a reporter fluorophore and a quencher. Cleavage of the probe by the exonuclease activity of Taq polymerase during strand elongation releases the reporter from the probe resulting in an increase in reporter emission intensity owing to its separation from the quencher. This increment in net fluorescence is monitored in real-time during each PCR amplification. The cDNA, will be used for real-time PCR performed in 48-well optical reaction plates with cDNA equivalent to 100ng RNA in a volume of 25 ml reaction containing Taqman Universal Master Mix (Applied Biosystem 4304437). Optimized concentrations of FAM-labelled probe and specific forward and reverse primers for HOXC11, HOXC12, SATB2, Osx and lncRNA HOTAIR (Applied Biosystem) from Assay on Demand. The results will be analyzed using a comparative method, and the values will be normalized to the GAPDH expression as endogenous controls (Borelli, et al. 2017).

Results

1. MMT Assay

The data of the MTT assay in MSCs treated with Osteoblast Medium (OsteoB), Lipogems Conditioned Medium (LG-CM) and LG-CM diluted with OsteoB (LGCM-OsteoB 1:1) are shown in the Figure 3. The viability of MSCs, was assessed considering the medium used in the different wells; No differences in the cytotoxic effect have been demonstrated in MSCs treated with OsteoB, LG-CM and LGCM+OsteoB compared with control.

2. alamarBlue

The cell proliferation has been studied with the alamarBlue assay. The MSCs cells were treated with three different medium sorted out for the experiments: OsteoB, LG-CM and LGCM-OsteoB (see paragraph 1) and the cells proliferation was followed at 7 and 14 days. The cells viability was always increased in the experiment with OsteoB, LG-CM, LGCM-OsteoB, compared with control. The rate of proliferation in MSCs treated with LG-CM and LGCM-OsteoB at 7 days, was higher 10% than control and OsteoB; at 14 days the proliferation increased more than of 30% (Figure 4).

3. HOXC11, C12, Osx, SATB2 and lncRNA HOTAIR Gene Expression

qRT-PCR was used to analyze gene expressions of HOXC11, HOXC12, SATB2, Osx and HOTAIR in MSCs using specific medium. The time and condition of the experiments were performed following the material and methods described above. Both, OsteoB and LGCM-OsteoB medium were able to regulate the expression of all genes object of the study. Moreover, the rate of hyper-expression was related to the medium used during the experiments; in MSCs stimulated with LGCM+OsteoB the up-regulation of the HOXC11, SATB2, Osx and HOTAIR genes was always higher than MSCs treated with OsteoB medium. The differences in the rate of the gene expression were related to the specific characteristic of the single gene studied. Conversely, the HOXC12 gene expression did not show appreciable differences in cells exposed to different treatment, compared with MSCs control (Figure 5-6).

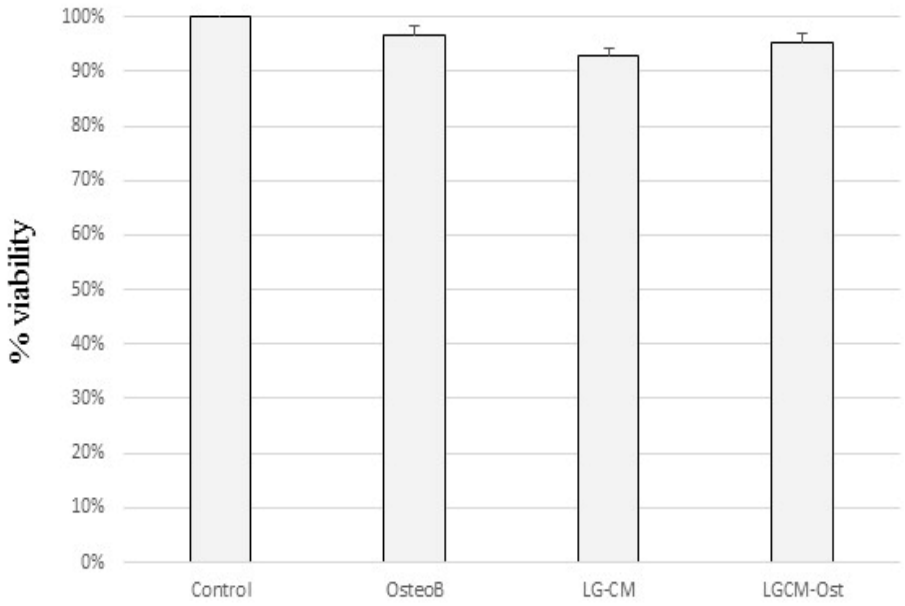

Figure 3. MTT assay did not show cytotoxic effects in MSCs treated for 24h with OsteoB, LG-CM and LGCM-OsteoB medium.

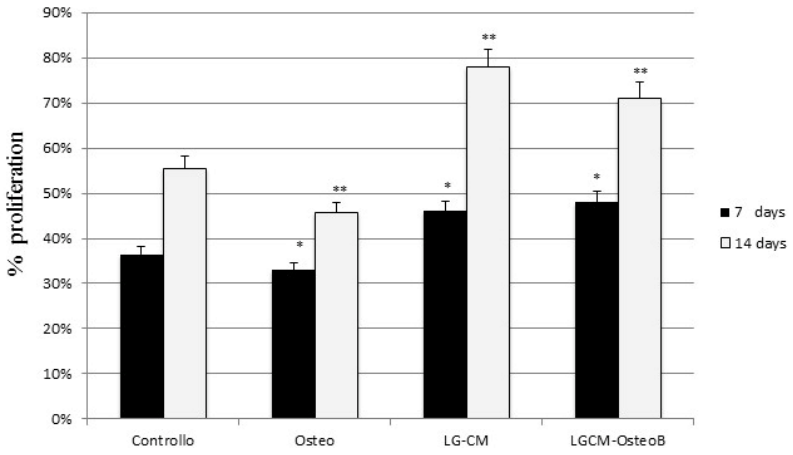

Figure 4. The proliferation of MSCs treated with OsteoB, LG-CM and LGCM-OsteoB medium for 7 and 14 days, was always increased during the experiments (see the text).

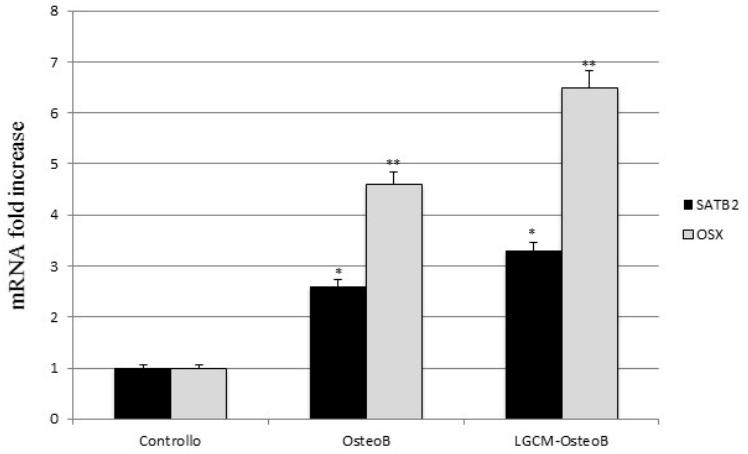

Figure 5. SATB2 and Osx gene expression was higher in MSCs treated with OsteoB and LGCM-OsteoB, compared with control (see the text).

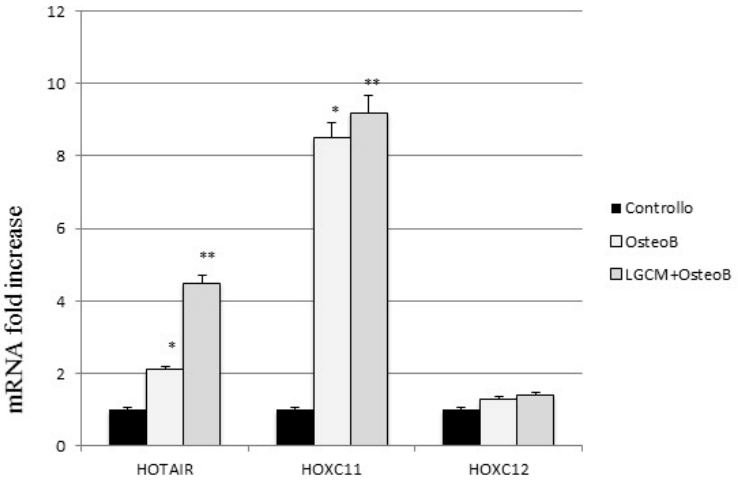

Figure 6. HOTAIR, HOXC11 gene expression was higher in MSCs treated with different medium than the control, while no HOXC12 gene expression was not influenced by OsteoB and LGCM-OsteoB medium.

Discussion

In the last years, the information about the role of Osteoblasts in the sorting out of bone tissue are increased. The bone remodeling process, is constantly controlled by osteoblastic lineage cells (Osteoclasts, Osteocytes). It has already demonstrated the commitment of osteoblastic lineage cells in the regulation of bone development, that play a key role like mechanic support for skeletal muscle; furthermore, bone matrix is a crucial reserve of Calcium and Phosphorus (Razzaque., 2009).

The osteoblasts development takes place through the transition from MSCs to pre-osteoblasts and, finally, osteoblasts. However, has not been clarified the molecular identity of the different stages; the characterization of these three steps provides a model to study the Osteoblasts differentiation. The molecular markers of mature Osteoblasts were already identified (Osteocalcin, Alkaline phosphatase, etc.); despite this, it is not clear which gene network regulates the transition from MSCs to pre-osteoblasts. Finally, the pre-osteoblast include a series of heterogeneous cells with intermediate characteristics between MSCs and osteoblasts, in this case is a lot of complex the identification of molecular markers able to lead the differentiation and development of mature Osteoblasts (Pritchard., et al. 1952; Owen., 1995; Owen and Machperson 1963). It has been shown the crucial role played by the specific transcription factors able to characterize different stages of Osteoblasts differentiation; these proteins work in a specific way during Osteoblasts lineage cell development and their activation is time-dependent.

Some of these Transcription factors have been already identified. Osx belong at family of homeobox genes located on chr 12q13.13, Osterix is one of the most regulator of osteoblasts differentiation (33 Long., 2011); this protein exerts its action inhibiting SATB2 gene expression. The SATB2 gene is a homeobox family member located on chr 2q32-33 and take part in the bone formation; its deregulation is related to several malformation like generalized osteoporosis, craniofacial dysmorphisms (cleft palate, mandibular hypoplasia and protruding incisor) (Leoyklang., et al. 2007; Rosenfeld., et al. 2009) .

Moreover, it has been demonstrated the role HOX gene family like must regulator of osteogenesis. Deregulation of Hoxd locus is related to the morphological anomaly of long bone development (Gonzalez-Martin., et al. 2014). The HOXA10 gene controls the Osteoblasts differentiation mainly by means of the Osteoblast related genes activation like Runx2, Alkaline phosphatase (ALP), Bone Sialoprotein (Spp) (Hassan, et al. 2007). Conversely, HOXA2 is an HOX protein inhibited by SATB2 and this regulation is related to a negative control of the osteogenesis (Zhang, et al. 2012).

Several studies suggested that HOTAIR is able to control the epigenetic regulation of DNA through a deep relationship with chromatin, involving the recruitment of complexes, which are able to identify specific sequences and induce silencing of several target genes. The HOTAIR epigenetically promotes Epithelial Mesenchymal Transition (EMT) mainly by means of silencing miRNA34a and interacting with Polycomb-Responsive-Element-2 (PRC2). In conclusion, based on the literature and the results obtained, it has been hypothesized that HOTAIR and HOX genes might induce the alteration of the epithelial cell memory program (Pdua AC., et al. 2013; Tano and Akimitsu et al. 2013; Liu, et al. 2015; Procino., et al. 2016)

In this study, we have demonstrated, for the first time, the involvement of HOXC locus and lncRNA HOTAIR during MSCs differentiation towards osteoblastic phenotypes. In general, in MSCs treated with LGCM-OsteoB and OsteoB medium obtained by adipose tissue with indirect method, we have always observed an up-regulation of HOTAIR and HOXC11 genes, but no differences in HOXC12 mRNA; the fold increase of HOTAIR and HOXC11 was higher in Mesenchymal Stem Cells treated with LGCM-OsteoB medium than MSCs treated with OsteoB (Figure 5-6). According with the literature, Osx and SATB2 genes expression were increased in MSCs treated with OsteoB and LGCM-OsteoB medium, compared with control. The hyper-expression of Osterix and SATB2 were always higher in MSCs treated with LGCM-OsteoB medium than MSCs exposed to OsteoB medium. Moreover, The positive regulation of Osterix gene was always related to the deregulation of SATB2 gene.

In our opinion, HOTAIR is a good candidate in the control of osteogenesis, on the basis of its position within the locus and the ability to regulate the HOXC

locus. In this paper we have shown, like HOTAIR is able to activate HOXC11 gene in MSCs treated with LGCM-OsteoB and OsteoB, conversely we did not observe none change in HOXC12 gene expression. On the other hand, we believe (on the basis of our results) that HOXC11 mRNA fold increase, is related to the up-regulation of Osx gene in MSCs exposed to a different medium; In turn, the hyper-expression of Osterix gene down-regulates SATB2 mRNA. Our theory is supported by the position within the genome of Osx (chr 12q13.13) close to HOXC cluster and SATB2 (chr 2q31.1) close to HOXD cluster, two area controlled by of HOX network and in particular by HOTAIR.

In conclusion, the data demonstrated the involvement of lncRNA HOTAIR, HOXC11 but not HOXC12, in the regulation of osteoblast related genes, although this is a preliminary study and further experiments will be useful in order to understand the role of HOX genes in the control of human osteogenesis.

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