RESEARCH ARTICLE

**HOXA9 is Underexpressed in Cervical Cancer Cells and its Restoration Decreases Proliferation, Migration and Expression of Epithelial-to-Mesenchymal Transition Genes**

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**Abstract**

HOX transcription factors are evolutionarily conserved in many different species and are involved in important cellular processes such as morphogenesis, differentiation, and proliferation. They have also recently been implicated in carcinogenesis, but their precise role in cancer, especially in cervical cancer (CC), remains unclear. In this work, using microarray assays followed by the quantitative polymerase chain reaction (qPCR), we found that the expression of 25 HOX genes was downregulated in CC derived cell lines compared with non-tumorigenic keratinocytes. In particular, the expression of HOXA9 was observed as down-modulated in CC-derived cell lines. The expression of HOXA9 has not been previously reported in CC, or in normal keratinocytes of the cervix. We found that normal CC from women without cervical lesions express HOXA9; in contrast, CC cell lines and samples of biopsies from women with CC showed significantly diminished HOXA9 expression. Furthermore, we found that methylation at the first exon of this gene could play an important role in modulating the expression of this gene. Exogenous restoration of HOXA9 expression in CC cell lines decreased cell proliferation and migration, and induced an epithelial-like phenotype. Interestingly, the silencing of human papilloma virus (HPV) E6 and E7 oncogenes induced expression of HOXA9. In conclusion, controlling HOXA9 expression appears to be a necessary step during CC development. Further studies are needed to delineate the role of HOXA9 during malignant progression and to afford more insights into the relationship between downmodulation of HOXA9 and viral HPV oncoprotein expression during cervical cancer development.

**Keywords:** Cervical cancer - HOX genes - HOXA9 - proliferation - migration - epithelial-mesenchymal transition

**Introduction**

Cervical Cancer (CC) is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases in 2012 (Ferlay et al., 2015). Development of this disease is closely associated with human papilloma virus (HPV) infection (Bosch and Munoz, 2002); however, it is estimated that only 0.03% of women who become infected with HPV proceed to develop CC (Sasagawa et al., 2012). Therefore, viral infection per se is not considered sufficient to generate a neoplastic process; it is, rather, a first alteration that predisposes cells to subsequent changes; if those do occur, the neoplasm is generated (Perez-Plasencia et al., 2008). It is evident that additional factors, such as genomic instability caused probably by viral oncogenes (E6 and E7), environmental factors, or the genetic background itself, are necessary for cervical tumorigenesis (Hyland et al., 2011). Alterations in developmental signaling pathways or transcription factors that regulate the ontogeny, such HOX genes, have recently been proposed as alternative secondary modifications that could lead to malignant transformation (Karamboulas and Ailles, 2013).

HOX genes in humans comprise a highly conserved family of 39 transcription factors that are grouped into four clusters: HOXA; B; C, and D, each group consisting of 13 paralog genes, with 9-11 members, distributed on the basis of sequence similarity and relative position within the group (Acaporpa et al., 1989). HOX genes play an important role in the delicate balance between cell proliferation and differentiation that is essential for normal fetal development during embryogenesis; the abnormal
function of these transcription factors has been implicated in human diseases, particularly in neoplastic processes (Castelli-Gair, 1998; Lappin et al., 2006). Thus, in recent years, much effort has been devoted to the study of HOX genes and proteins and their link with cancer (Shah and Sukumar, 2010). In cervix, despite some progress with the modulation of HOX member in cervical cancer (Amali et al., 1999; Hung et al., 2003; Lopez et al., 2006b; Gonzalez-Herrera et al., 2015), the participation of these transcription factors in cervical carcinogenesis has not been explored in depth. In this study, we evaluated HOX gene deregulation in CC; in particular, we focused on HOXA9 modulation and whether this phenomenon results in an advantage for the CC cell.

Materials and Methods

Cell culture

HeLa, SiHa, and C-33A cell lines derived from CC, as well as the non-tumorigenic keratinocyte HaCaT cell line, were obtained from the generosity of the German Cancer Research Center (DKFZ, Heidelberg, Germany). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing GlutaMAXTM and supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin, and 100 μg/ml Streptomycin, at 37°C with an atmosphere of 5% CO2 and 90% relative humidity (all of the previously mentioned products were acquired from Gibco®, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were propagated according to the methods recommended by the suppliers.

Cervical samples and HPV detection

Informed consent was obtained from all individual participants included in the study (Prot.R-2012-785-090). Patients were recruited at the Western Medical Center of the Mexican Institute of Social Security (IMSS) and cervical scrapes of clinically healthy women without HPV infection were collected. Cervical samples and HPV detection was performed as follows: genomic DNA was extracted from all samples and screened by conventional PCR utilizing the PGMY 09/11 primers, as described elsewhere (Gravitt et al., 2000), and samples that were positive to HPV were subsequently analyzed with the Linear Array HPV Genotyping Test (Roche Applied Science, Penzberg, Germany) (Coutlee et al., 2006).

Isolation of primary keratinocytes

Primary Keratinocytes (Kers) were obtained from the normal cervixes of women who underwent hysterectomy, and the samples were taken under signed informed consent at the Western Medical Center - IMSS. Biopsies were collected in EPI-LIFE CF medium (Thermo Fisher Scientific, Inc.) with 100 U/ml Penicillin and 100 μg/ml Streptomycin. After removal of connective and fatty tissue, the biopsy was chopped into 0.5-cm2 fragments and treated with 25 U/ml collagenase (Sigma-Aldrich Quimica, S. A. de R.L. de C.V., Toluca, México) and 25 U/ml dispase (Thermo Fisher Scientific, Inc.) overnight at 40°C with constant movement. Then, the epidermis was removed from connective tissue with tweezers. Epithelial cells were disrupted by incubation for 30 min at 37°C in 2 ml of 0.25% trypsin-EDTA solution (Thermo Fisher Scientific, Inc.). Trypsin was neutralized with FBS and the cells were collected by centrifugation and resuspended in 13 ml of selective medium for keratinocytes EPILIFE CF supplemented with Human Keratinocyte Growth Supplement containing: 0.2% v/v pituitary gland extract (BPE), 5 μg/ml bovine insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml bovine transferrin, 0.2 ng/ml human Epidermal Growth Factor (EGF), and Gentamicin/Amphotericin (all from Thermo Fisher Scientific, Inc.). The cultures were maintained at 37°C in a humidified atmosphere with 5% CO2.

RNA isolation and expression microarrays

Total RNA from the different cell lines, cervical scrapes, and biopsies was extracted with the NucleoSpin RNA Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer’s instructions. RNA was quantified by absorbance at 260/280 nm. For microarray analysis, RNA quality was determined using an Agilent 2100 Bioanalyzer and the RNA 6000 NanoChip Kit (Agilent Technologies, Santa Clara, CA, USA). Double stranded complementary DNA (cDNA) was generated from 10μg of RNA using the cDNA Synthesis Kit System (Roche Applied Science) and purified with the GenEluteTM PCR Clean-Up Kit (Sigma-Aldrich Quimica, S. A. de R.L. de C.V.); thereafter, the cDNA was labeled with Cy3 and hybridized in a Human Gene Expression Array 12x135K (Roche Applied Science). Microarrays were scanned on the MS200 Scanner and the data obtained was processed using DEVA ver 1.2 software (Roche Applied Science). Fluorescence intensities were normalized using the RMA algorithm and the information was subsequently analyzed with CLC Main WorkBench ver. 7.0.3 software. Genes that were differentially expressed with a fold-change >1.5 and that exhibited a permutation p-value <0.05 were selected.

RT-PCR and qPCR assays

cDNA synthesis was performed from 5 μg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) primed with Oligo dT. The cDNA obtained was employed in a quantitative Polymerase Chain Reaction (qPCR) to evaluate gene expression levels utilizing the LightCycler® FastStart DNA Master PLUS SYBR Green I Kit with 2.0 LightCycler technology (Roche Applied Science) under conditions specified by the manufacturer. The gene-specific primers (Supplementary Table 1) were designed with Oligo Primer Analysis ver. 6.0 software (Molecular Biology Insights, Inc., Colorado Springs, CO, USA) from sequences obtained at the Entrez Nucleotide Database of the National Center of Biotechnology Information (NCBI). Quantification of PCR products was calculated employing Light Cycler ver. 4.1 software and was calculated using the following reference genes: GAPDH; ACTB; RPLP0, and RP18S.
Two types of analysis were used: 2ΔΔCT and ΔCT. A melting curve analysis was also performed for each gene to determine the specificity of the reaction.

**CpG methylation detection assay**

To determine the presence of CpG islands, analysis of 1,400-bp upstream and 800-bp downstream of translation start was performed using the MethPrimer online tool (Li and Dahiya, 2002). Additionally, localization of restriction sites for Mspl and HpaII restriction enzymes was determined utilizing CLC Main Workbench ver. 7.0 software (Qiagen, Germantown, MD, USA). Specific primer pairs were designed using Oligo Primer Analysis ver. 6.0 software (Molecular Biology Insights, Inc.) to amplify five different zones (Z1-Z5), which cover the different CpG islands found (Supplementary Table 2, Figure 3a). Genomic DNA from cell lines and primary keratinocytes was extracted with the QIAamp DNA Mini Kit (Qiagen) and digested with Mspl restriction enzyme, and its isoschizomer non-methylation-sensitive HpaII (New England Biolabs, Inc., Ipswich, MA, USA) at 37°C for 1 h. The digested DNA was used as template for PCR amplification reactions utilizing the Expand High Fidelity enzyme (Sigma-Aldrich Quimica S. de R.L. de C.V.) as recommended by the manufacturer. The amplification products were separated by electrophoresis in 2% agarose gels, visualized, and digitalized for analysis employing a DigiDoc-It system program (UVP, LLC. Upland, CA, USA).

**Immunohistochemistry**

Histologically normal cervical epithelium (35) and CC (21) samples that were paraffin-embedded were utilized to construct a Tissue MicroArray (TMA) using the Tissue Microarray ATA 100 (Chemicon, Temecula, CA, USA). TMA slices were used for automated immunodetection assays utilizing the Ventana BenchMark System (Roche Applied Science), the anti-HOXA9 antibody (cat. Ab922565; Abcam, Cambridge, MA, USA), and the ultraView Universal DAB Detection Kit (Roche Applied Science). HOXA9 expression was evaluated by a Pathologist and classified according to level of positivity as low, medium, or high.

**Plasmid constructs**

The commercial HOXA9 Open Reading Frame (ORF) (eDNA clone MGC: 19648; Thermo Fisher Scientific, Inc.) was subcloned into the pLVX-tight-Puro vector, a tetracycline-inducible expression system, and into the pLVX-Puro vector, a constitutive expression system (Takara Bio, Inc., Kusatsu, Shiga, Japan). Lentiviral particles were produced by transfecting Lenti-X™ 293T cells with Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) and Lenti-X™ HT Packaging Systems (Takara Bio, Inc.), according to the manufacturer’s instructions. Lentiviral production was checked with Lenti-X™ GoStix™ (Takara Bio, Inc.) and the titer was determined by Enzyme-Linked Immunosorbent Assays (ELISA) with the Lenti-X™ p24 Rapid Titer Kit (Takara Bio, Inc.), according to the supplier’s instructions. Then, 4 x 10⁵ cells were transduced with at least 5 x 10⁴ IFU viral particles. Positive selection was performed by adding G418 to final concentration of 500 μg/ml (Takara Bio, Inc.) or Puromycin to a final concentration of 1μg/ml (Sigma-Aldrich Quimica S. de R.L. de C.V.).

**Clonogenicity assays and colony formation**

Cell culture plates (p60) were seeded with 5 x 10⁴ cells in 3 ml of medium; after 24 h of culture, the plates were infected with 2 x 10⁵ Inclusion-Forming Unit (IFU) of supernatant containing viral particles (LVX-Puro-HOXA9 and LVX-Puro empty virus); as control, one plate remained uninfected. At 48-h post-infection, Puromycin was added to cultures at a final concentration of 1 μg/ml. Five days later, the cells were fixed with 3.7% paraformaldehyde, stained with 0.05% crystal violet, and photo documented. Assays were performed in triplicate. This infection scheme was also evaluated by using the xCELLigence system (Roche Applied Science).

**Cell proliferation and viability assays**

The cell proliferation of HOXA9-expressing HeLa cells (in the presence of Doxycycline) was assessed with the xCELLigence system platform (Roche Applied Science). Briefly, 2.5 x 10⁴ cells per well were seeded on a 96-plate E-Plate wells and introduced into the xCELLigence reading station. After 24 h, Doxycycline was added to the culture cells to induce HOXA9 expression; proliferation was assessed in real time every 30 min during 72 h. Three independent experiments were performed with four duplicates in each case. Cell viability was analyzed by the cleavage of tetrazolium salt Water Soluble Tetrazolium Salts (WST-1) to formazan (Roche Applied Science) by reading the absorbance at 440 nm on a microtiter plate reader (Synergy™ HT Multi-Mode Microplate Reader; Biotek Instruments, Inc., Winooski, VT, USA). The value of HOXA9 uninfected cells (HeLa TET) was employed as 100% cell survival.

**Migration assays**

Hela-LVX and HeLa-LVX-HOXA9 cells were seeded onto a p60 plate (5 x 10⁴ cells in 3 ml of medium); after 24 h of culture, the surface of each plate was gently scratched with a cell scraper; cells were washed with PBS, and 3 ml of fresh DMEM medium was added. Photographs were taken at 0, 24, and 48 h to evaluate the scratches. The photographs were analyzed with ImageJ ver. 1.49 software and the Wound Healing Tool plug-in, which evaluates the wound area (implying the area not covered by cells). The data obtained were graphed employing Microsoft Excel.

**Apoptosis detection**

Apoptosis detection was performed by Flow Cytometry (FC) using Annexin-V-FLUOS/Propidium iodide (Roche Applied Science) as markers of cell death. In brief, cells were treated with cold PBS/EDTA and harvested by scraping, packed by centrifugation and washed twice with PBS. Apoptotic cells were incubated for 15 min at room temperature with Annexin-V-FLUOS in combination with Propidium Iodide (PI). The percentage of positive cells was determined by FC using an Attune Cytometer (Thermo Fisher Scientific, Inc.).
Silencing of E6 and E7 from HPV18 in HeLa cells

The following sequences were employed to silence HPV18 E6 and E7: GAT CGG CTA ACA CTT GTA AGT TTT TTT (E6/E7 1), and AAT TCA AAA AAC ATT TAC CAG CCC GAC GAG TCT TTT GAA CTC GTC GGG CTT GATA ATT GGC (E6/E7 2); these were previously described elsewhere (Qi et al., 2010). These sequences were cloned into the pLVX-shRNA1 vector (Cat. # 63217; Takara Bio, Inc.) transfected in HEK cells to produce lentiviruses. The shE6/E7-1 and shE6/E7-2 lentiviruses were utilized to transduce HeLa cells. After infection, cells were selected by adding Puromycin to the cell culture medium.

Statistical analysis

All experiments were performed in duplicate and repeated independently at least twice. The results are expressed as means ± Standard Deviation (SD). For differences between groups, analysis was performed using unpaired, two-tailed Student t-test and statistical significance at set at p<0.05.

Results

Altered HOX Gene Expression Between Non-tumorigenic Keratinocytes (HaCaT) and CC-derived Cells (HeLa)

With the aim of determining HOX genes differentially expressed in CC-derived cells, a genetic expression comparison between HeLa (CC-derived cell line) and HaCaT (non-tumorigenic keratinocyte derived cell line) using a microarray-based platform (see Materials and Methods) was performed. Variations in the expression levels of HOX genes were calculated taking the expression value of 0.09. This gene is involved in the regulation of endothelial cell proliferation and has been observed that controls have lower ΔCp values, indicating expression is higher in controls than in CC samples, nine samples of biopsies from patients with CC and 18 cervical scrapes from women without obvious cervical lesions were recruited (Figure 2b). HOX9 mRNA levels were analyzed and expression values were calculated for each patient by ΔCp analysis, that is, the Cp of the HOX9 gene minus the expression of a reference gene from the same patient; from this analysis, it was observed that controls have lower ΔCp values, indicating that HOX9 expression is higher in controls than in HeLa (0.06-fold), and SiHa (0.38-fold) (Figure 2a). With the purpose of knowing whether downregulation of HOX9 is a common characteristic of CC cells, the expression of this gene was analyzed by qPCR in additional CC-derived cell lines: SiHa (positive to HPV16), and C-33A (negative to HPV infection). Because SYBR green-based detection was used for qPCR, the melting curves of the amplicons were determined to discard unspecific amplifications, and the expected Molecular Weights (MW) of qPCR products were also confirmed on agarose gels (Figure 2a). The relative expression of HOX9 was determined in HeLa, SiHa, and C-33A cells using the expression of HaCaT as calibrator. A significant decrease in HOX9 levels was observed in the three tumorigenic cell lines, with C-33A the cell line showing lowest expression (0.0099-fold), followed by HeLa (0.06-fold), and SiHa (0.38-fold) (Figure 2a).

CC biopsies showed lower expression of HOX9 mRNA than control cervical cells

To determine whether the diminished expression of HOX9 observed in CC-derived cell lines is also present in CC samples, nine samples of biopsies from patients with CC and 18 cervical scrapes from women without obvious cervical lesions were recruited (Figure 2b). HOX9 mRNA levels were analyzed and expression values were calculated for each patient by ΔCp analysis, that is, the Cp of the HOX9 gene minus the expression of a reference gene from the same patient; from this analysis, it was observed that controls have lower ΔCp values, indicating that HOX9 expression is higher in controls than in HeLa.
patients with CC. These differences were statistically significant and correlated with our previous results.

The HOXA9 Promotor Is Methylated in CC Cell Lines, But Not in Keratinocytes and HaCaT Cells

To determine whether the expression of HOXA9 is reduced in tumor cells due to hypermethylation, as was previously described in other tissues, the methylation status of five different zones belonging to four CpG islands located within the promoter and gene sequence of HOXA9 (-1,400pb to +800pb) was evaluated by restriction with methylation-sensitive and -insensitive restriction enzymes (HpaII and MspI, respectively), followed by PCR (Figure 3a, see Materials and Methods). As depicted in Figure 3b, HaCaT cells demonstrated the methylation of Zones 1 and 5, localized at the ends of the analyzed area, but Zones 2, 3, and 4, which correspond to the near promoter region and to the first exon, were not methylated. In contrast, HeLa and C-33A cells exhibited methylation in all analyzed zones (Z1-Z5). Interestingly, SiHa cells have a pattern similar to that of HaCaT cells (Z1- and Z5-methylated) with some methylation in Z2, which corresponds to the promoter region. As control, the methylation status of normal cervical primary keratinocytes isolated from a patient without cervical lesions and without HPV infection, was also included; normal keratinocytes demonstrated nearly no methylation in all analyzed zones.

Figure 2. HOXA9 Expression in Normal Cervical Cell vs. CC-derived Cells. (a) In the left panel, representative graphics of the amplification curves obtained from HOXA9 and the reference gene GAPDH are depicted for HaCaT, HeLa, SiHa, and C-33A cell lines. Agarose gels of PCR products and melting peaks are also depicted. Right panel shows a graphic of HOXA9 relative expression (2ΔΔCp); the values depicted were normalized to three reference genes (GAPDH, ACTB, and PGK1) and were determined relative to HaCaT. (b) Relative HOXA9 mRNA expression depicted as ΔCp in cervical scrapes of healthy women (CTRL) and cervical biopsies with Cervical Cancer (CC Biopsies). ΔCp was calculated by subtracting the Cp value of the reference gene (GAPDH) of one sample from the Cp obtained for the HOXA9 gene of the same sample. The graph depicts medians (dark lines), 5-95 percentile limits (boxes), and InterQuartile Ranges (IQR) (Whiskers). Two-tailed Student t -test (p <0.001). Tables right. Information on the samples of controls: sample number and ΔCp obtained in the analysis. Information of patients with CC biopsies: patient number, HPV genotype positivity, and ΔCp

Figure 3. Methylation Status of HOXA9 and HOXA9 mRNA Regulation by HPV18 E6/E7 Oncogenes. (a) Representative image of the sequence: -1,400bp to +800pb from the transcription start of the HOXA9 gene, predicted CpG islands, PCR-amplified regions (Z1-Z5), and restriction sites (MspI/ HpaII) are depicted as dark boxes, horizontal lines, or vertical light-grey lines, respectively. (b) Gel electrophoresis of the amplification products for Z1-Z5 after restriction with MspI or HpaII for HaCaT, HeLa, SiHa, and C-33A cell lines and normal primary Keratinocytes (Kers). (c) Relative HOXA9 expression in HeLa cells transduced with shRNA coding virus directed to E6/E7 of HPV18 (shE6/E7-1 and shE6/E7-2), and irrelevant shRNA was also used as control (shIrre). Expression values of E6/E7 and HOXA9 were calculated relative to the expression observed in HeLa-shIrre. For normalization, reference genes RPL32, RP18S, and RPLP0 were used.

Figure 4. Immunodetection of HOXA9 in Normal Cervical and Cervical Cancer (CC) tissues. CC biopsies and normal cervical tissues were used to detect HOXA9 by immunohistochemistry with an anti-HOXA9 antibody. Representative images are presented at a magnification of 10X. HOXA9 expression was evaluated and classified according to degree of positivity: negative; low; medium, or high, and the percentages are included at the bottom of the image.
### Supplementary Table 1. Primer pairs utilized for PCR and qPCR

| GENE | GenBank ID | Sequence | Size |
|------|------------|----------|------|
| HOXA9 | NM_152739.3 | F- TTGTCCCCGTAGCTGACCTGCT R- GTCGCTTGAGGTAGCTGAC | 249pb |
| ACTB | NM_001101.3 | F- CGGCTTCTTTCTTGCTCCCAACT R- CGAGAAAAGCCCGCTTCGCC | 316pb |
| GAPDH | NM_002046 | F- CACTGCCACCAGAAGACTGTT R- TGTAGGCCATGAGCGCTTCA | 449pb |
| RPS18 | NM_022551.2 | F- CGTAGGGCCGCGGAAAA R- CAGTGTG GCCCGTCTTCAAGG | 283pb |
| RPLP0 | NM_001002.3 | F- CCTCATATCCGGGGAAGTGT R- GCAGAGCTGGCCACCTTAAGTG | 95pb |
| HOXA9 ORF | NM_152739.3 | F- CAGTTTCTAAATTTCCGTGGTC R- AGTCGCTTGGAAGTTGAACTCTT | 1011pb |
| HOXA11 | NM_005523.5 | F- CTTCCCGCCCAACTGAGGACAC R- GCCGCTGAGCTTCTAAGGAGAG | 281pb |
| HOXB3 | NM_002146.4 | F- CGAGCACCAACTCCACCTCA R- TCTTGAGCCTCCGGCTGTA | 445pb |
| HOXB4 | NM_024015.4 | F- CGCTCCACTCCGCGTGCAA R- TCCATCTATGCGCCGGTGTC | 261pb |
| HOXB5 | NM_002147.3 | F- CCGGCTCTTCACTGAGCTTC R- AGCGGTTAGTGTTGAACCTCT | 242pb |
| HOXB6 | XM_005257284.2 | F- CTGGTCTCCGCGGACAGAGAGAA R- TGCAGAGCGCGTGACAGTT | 297pb |
| HOXB9 | NM_024017.4 | F- CCAGGCCGCTCTTCAATCAAAG R- TTGGCCTCTTCTATTCAATT | 331pb |
| HOXB13 | NM_006361.5 | F- AACCAGGCCAGATCTCCTGTG R- GGCGCTCCGAGAGCTCCGTG | 306pb |
| HOXC12 | NM_173860.1 | F- AGCCAGGCCGCTCTTCACTG R- AAATCGCAGCCCTTGCTTTC | 226pb |
| HOXC13 | NM_017410.2 | F- TTGGCGCGCTCTTGAGATGC R- TTAAATCGCTAGCCGCGTATT | 300pb |
| HOXD10 | NM_002148.3 | F- CCTCGGTCTCCAGCTCCGGAAG R- AGGGTTGGCGTCAGCTCTTC | 306pb |
| HOXD11 | NM_021192.2 | F-GGGGGCAGCAGAGAGCACGAC R- GGCTGGAGGAGAGCTGGAGAA | 286pb |
| CDH1 | NM_001317184.1 | F- CCGGATCGCTGAGCTTGGCAAG R- TGCCCTGCAGGCGGCTC | 271pb |
| GRHL2 | NM_024915.3 | F- GAAGAGAGAGCAGAAGAGAG R- CCACAAACCAGGCTGATTTC | 297pb |
| TCF4 | NM_001083962.1 | F- TCAGAGAGGACAGAAATATTAG R- CCGAGCCTTGGATTC | 321pb |
| TWIST1 | NM_000474.3 | F- CCGGTCCCACCTCAGC R- TTGGCTGATGGGACAGCCTC | 393pb |
| WNT5A | NM_003392.4 | F- TTGGTGGTCGCTTGTATGGAAT R- ACCTCTGATGTCAGAATGATT | 203pb |
| WNT5B | NM_032642.2 | F- GGGGAGGAGAGAGAGAGAGAG R- GGCGCTGCGGCTCCACAT | 319pb |

### Supplementary Table 2. Primer pairs utilized for amplification of HOXA9 promoter CpG islands

| POSITION | SEQUENCE | SIZE |
|----------|----------|------|
| Z1 1655-1906 | F- CCGTGGTACCTCCTCCGGCGAGTCR- GTTGCTGGGCTCCCTCAGTCA | 251pb |
| Z2 2051-2369 | F- GCCTGGCGCTTGGGAATCTCTGAR- GGCTGGGCGCCAGATCACCTGT | 514pb |
| Z3 2951-3202 | F- GCAGGACAGCTCCCTTACATCA R- CACCTCGCCCTGGAATTCGGAA | 455pb |
| Z4 3376-3486 | F- CTCCAGTCCCGAAGCGAGGT R- TCCCGGCGGCTATTCTAC CAGTCR- GGTGCGGCG GCCCGTGAC | 386pb |
| Z5 3689-722 | F- GTCCCCCAGCTTACGACAC TACR- CGTCCAAACACCATATTG | 298pb |
HOXA9 Protein Expression Was Diminished in CC Tissues

To corroborate these previous observations, HOXA9 expression was evaluated by immunohistochemistry in biopsies from patients with CC, and also in healthy cervical samples as control. The HOXA9 signal was evaluated by a Pathologist and classified as negative, low (+), medium (++) and high (+++), according to the positivity. The percentages of different degrees of HOXA9 expression were calculated and represented graphically (Figure 4). It was found that 8.57% of normal cervical-tissue samples have high HOXA9 expression, 62.8% medium expression, and 28.57% low expression; instead, in CC samples, high HOXA9 expression decreases to 4.76%, medium expression decreases to 57.14%, and low expression increases to 38.09%. These results indicate that protein expression of HOXA9 has a tendency to decrease in CC when compared with normal cervical tissues.

Exogenous HOXA9 Expression Induced Lower Colony Formation in CC-derived Cell Lines and Modified the Cellular Phenotype

The questions that arose from the previous observations including the following: why was the expression of HOXA9 diminished in CC-derived cell lines and in CC biopsies, and was diminution of HOXA9 an advantage to cancer-cell establishment. To determine whether the presence of HOXA9 is compatible with cell growth and proliferation, expression of HOXA9 was exogenously restored in CC-derived cells. For this, lentiviral particles carrying a Puromycin-resistant gene (LVX) and the Puromycin-resistant plus HOXA9 gene (HeLa LVX-HOXA9) were produced; thus, only infected cells would be able to survive and form colonies when cultured in the presence of Puromycin. As observed in Figure 5a, cells that were not infected did not survive and no colony formation was observed; instead, cells infected with viral particles carrying only the Puromycin-resistant gene (LVX) formed a great number of colonies. However, cells infected with...
viral particles carrying the Puromycin-resistant gene and HOXA9 (LVX-HOXA9) formed low numbers of colonies (Figure 5b), indicating that the presence of HOXA9 affects the growth of CC-derived cells.

In addition, it was observed that infection with exogenous HOXA9 not only decreased the number of colonies formed, but also changed cellular morphology, that is, HeLa cells, which normally grow isolated with no visible cell junctions and with mesenchymal morphology, in presence of HOXA9 cells have larger and irregular borders with indicia of cell junctions and epithelial morphology (Figure 5c). Due to the observation of such changes, we decided to evaluate modifications in the expression of genes involved in the epithelial-to-mesenchymal transition (CDH1, GRHL2, TCF4, TWIST1, WNT5A, and WNT5B) after restoration of HOXA9 (Figure 5d). We found that genes such as GRHL2, which is normally present in epithelial, and not in mesenchymal cells, increase their expression when HOXA9 is restored. Likewise, the TCF4 and TWIST1 genes, which are related with the mesenchymal phenotype, decrease their expression 0.75-fold and 0.79-fold, respectively (Figure 5e).

**Restoration of HOXA9 Expression Decreases Cell Proliferation and Cell Metabolism, But Does Not Induce Apoptosis in CC-derived Cell Lines.**

The results of clonogenic assays suggest a modulation of cell proliferation and viability by HOXA9. This hypothesis was further evaluated by measuring the cell growth of HeLa cells infected with viral particles carrying the Puromycin-resistant gene alone or with HOXA9. Cell growth was evaluated in real time using the Real-Time Cell Analyzer (RTCA) xCELLigence System. Cells were seeded, infected with viral particles and, after 72 h, Puromycin was added to the culture. As presented in Figure 6a, uninfected HeLa cells were not able to survive and died at 120 h of culture. Cells infected with lentiviral particles carrying the Puromycin-resistant gene only (LVX) exhibited growth curves that indicated more surviving cells; in turn, cells infected with the Puromycin-resistant gene and HOXA9 (LVX-HOXA9) displayed growth curves indicating a lower number of surviving cells in comparison with those shown with LVX alone. These results corroborate the negative effect that HOXA9 exert on the growth of the CC-derived cell line. Employing another strategy, a conditional model (TETracecline-controlled Transcriptional Activation [TET] system) was additionally developed, in which HOXA9 expression is controlled by the presence of Doxycycline in the culture; two different clones were obtained. Using this system, cell proliferation was also evaluated with and without the inducer of HOXA9 expression. As displayed in Figure 6b, induction of HOXA9 reduced cell proliferation in both clones.

Taking advantage of the previously developed conditional model, cell metabolism was also evaluated by using WST-1. As depicted in Figure 6c, we found that HOXA9 expression strongly decreases cell metabolism; similar results were also obtained using the SiHa cell line (data not shown).

**Exogenous HOXA9 Expression Decreases the Migration Rate of HeLa Cells**

Another feature of cancer cells is an increased ability to migrate. In order to test whether exogenous HOXA9 expression modulated CC-cell migration, scratch assays were performed on cultures of HeLa cells infected with LVX-Puro (viral particles only with the Puromycin gene) and with LVX-HOXA9 cells (viral particles with the HOXA9 and the Puromycin gene). Time for wound-closure was evaluated at 0 h, 24 h, and 48 h. As can be observed in Figure 6d, cells expressing HOXA9 migrate more slowly than cells without it. Images of cell growth were analyzed by the “ImageJ” computer program to calculate the uncovered area of the wound. These analyses showed that, although initially (0 h), the wound area of HeLa-LVX-Puro cells was slightly larger, it decreased faster than the wound area of HeLa-LVX-HOXA9.

Because HOXA9 decreases colony formation, cell proliferation, cell migration, and cell metabolism, it was in our interest to evaluate whether HOXA9 also modulates the apoptosis process. The percentage of apoptotic cells in the presence or not of HOXA9 was evaluated and no differences between early and late apoptosis, comparing Hela-LVX vs. Hela-LVX-HOXA9 and HeLa-TET vs. HeLa-TET-HOXA9 were observed (data not shown).

**Discussion**

HOX genes encode transcription factors that regulate and determine different cell types during embryonic development (Castelli-Gair, 1998). They are considered as master regulators involved in fundamental processes during the development of an organism (Svingen and Tonissen, 2006). In recent years, abnormal expression of these genes has been linked with the emergence of various, solid and hematopoietic malignancies (16-20). In CC, there are evidences concerning the overexpression or downregulation of HOX members, indicating its possible role during cervical carcinogenesis (Lopez et al., 2006b; Gonzalez-Herrera et al., 2015). In order to underscore differences in HOX genes expression in CC and to corroborate and complement previous findings, we performed a comparison by microarray of the mRNA expression profile of two cell lines: one derived from CC (HeLa), and the other, from non-tumorigenic keratinocytes (HaCaT). A differential expression of HOX3, HOX9, and HOX11 genes in HeLa compared with HaCaT cells was found. The HOXA cluster has been related with normal development of urogenital tracts (Burel et al., 2006); in particular, HOXA9 and -11 are important in controlling the embryonic development of Müller ducts (Hsieh-Li et al., 1995). With regard to HOXA9, this gene has been found overexpressed in hematological malignancies (Milne et al., 2010)(Zhao, 2014 #203), but in solid tumors, especially epithelial tumors, it has been observed as downregulated (Gilbert et al., 2010; Chen et al., 2012; Hwang et al., 2015). These observations were in agreement with our observations. We found that HOXA11 was slightly upregulated in Hela cells compared with HaCaT. The expression of HOXA11 was found unchanged.
in normal and malignant cervical cells (Lopez et al., 2006b). A report from Bai et al. indicates that HOXA11 is regulated by methylation and that the expression of this gene decreases in gastric cancer; moreover, these authors observed that silencing of HOXA11 induced cell proliferation of tumor cells (Bai et al., 2014).

Concerning the HOX B locus, we observed a modulation of HOXB3, -4, -5, -6, -9, and -13, and of these, only HOXB4 was found to be downregulated, while the remaining genes were overexpressed. According to our results, overexpression of HOXB genes has been observed in CC (Lopez et al., 2006a). Lopez et al. found the expression of HOXB1, -B3, -B5, -B6, -B7, -B8, and -B9 genes in normal cervix epithelia and in SCC. Moreover, the authors found the expression of HOXB2, -B4, and -B13 only in tumor tissues. We did not find variations in the expression of HOXB2 and observed downregulation of HOXB4, perhaps due to the different approach and the targets measured (we measured mRNA and Lopez et al., protein). Despite these dissimilarities, we also found upregulation of HOXB13. This phenomenon has also been observed in other works (Lopez et al., 2006b; Gonzalez-Herrera et al., 2015) (Gonzalez-Herrera, 2014 #204), and in other cancer types (Zhu et al., 2014; Barresi et al., 2015) (Long, 2013 #205). Regarding the HOXC cluster, we observed reduced expression of HOXC12 and -C13 in the CC-derived cell line HeLa. HOXC12 was recently proposed as a tumor suppressor gene downregulated by methylation in head and neck SCC (Guerrero-Preston et al., 2014). HOXC13 regulates the expression of keratins, and abnormal expression of this gene has been observed in malignant pilomatrixoma (Jave-Suarez et al., 2002; Cribier et al., 2006); in SCC, expression of HOXC13 is augmented and modulated by histone methylation (Marcinkiewicz and Gudas, 2014b; Marcinkiewicz and Gudas, 2014a). Overexpression of HOXC13 was additionally observed in melanoma (Cantile et al., 2012), and functional studies indicate that overexpression of HOXC13 induces proliferation and tumorigenesis; conversely, silencing of this gene induces apoptosis and cell cycle arrest (Kasiri et al., 2013).

HOXD10 was observed with lower expression in HeLa cells compared with HaCaT, and the expression of HOXD11 remains unchanged in both cell lines. Posterior HOXD and -A (9-11) genes have redundant functions in reproductive organ development, and mutations in these genes induce changes in the epithelia, from columnar to squamous, and promote the migration of myeloid suppressor cells (Raines et al., 2013). In this regard, downmodulation of HOXD10 and HOXA9 could be involved in the maintenance of the epithelial phenotype and in the modulation of the immune response. The discrepancies between our data and those previously reported in relation to HOXB4, HOXC12, and HOXC13 may be due to the different techniques used, as well as to the new isoforms recently described (Brunskill and Potter, 2012).

Particularly with HOXA9, we observed that the proximal sequence (-200 bases) at the transcription start and the following (+150) bases after this, comprises an area of critical importance for the regulation of HOXA9 mRNA, and that it is a principal area of methylation. Our observations are in agreement with those shown in other models of epithelial malignancies; methylation and HOXA9 loss of expression have been reported in breast, ovarian, bladder, oral, and lung cancer (Rauch et al., 2007; Wu et al., 2007; Guerrero-Preston et al., 2011; Kim et al., 2013; Conway et al., 2014). All of these observations indicate a possible role of HOXA9 as a tumor suppressor gene in epithelial cells; unlike its well described oncogenic potential in hematopoietic malignancies (Collins and Hess, 2015).

The tumor suppressor function of HOXA9 has been extensively investigated in breast cancer, where it has been shown that HOXA9 directly regulates BRCA1 and other genes involved in invasion, growth, and metastasis (Gilbert et al., 2010). Uchida et al. observed that high methylation levels of HOXA9 are associated with a high risk of metastatic tumors; in addition, the authors found that reduced HOXA9 expression confers an advantage on cell growth (Uchida et al., 2014); these observations are consistent with our results in CC-derived cells. We observed that restoration of HOXA9 expression in CC-cells lines infected with HPV 18 significantly decreases cell growth, colony formation, metabolism, and cell migration. Contrary to our results, Sun et al. observed high expression of HOXA9 in laryngeal SCC compared with normal mucosal cells; also, using liver hepatocellular cells, these authors demonstrated that downregulation of HOXA9 inhibits cell growth (Sun et al., 2013). Conversely and in agreement with our results, Uchida et al. observed that re-expression of HOXA9 in oral cancer cell lines inhibits cell growth (Uchida et al., 2014). The different tissues employed in those experiments could explain the controversial observations; hepatocellular cells are different from oral cancer cells, which derive from squamous epithelial tissue, as do CC cells.

We also observed that HOXA9 restoration induces morphological changes that resemble the epithelial phenotype. Because HOXA9 regulates the expression of cadherins (VE-Cadherin and P-Cadherin) (Rossig et al., 2005; Ko and Naora, 2014), we evaluated the expression of E-cadherin (CDH1), together with other important markers of epithelial-to-mesenchymal transition. The expression of CDH1, TCF4, and TWIST1 was slightly increased in CC cells, these authors demonstrated that downregulation of HOXA9 inhibits cell growth (Sun et al., 2013). Conversely and in agreement with our results, Uchida et al. observed that re-expression of HOXA9 in oral cancer cell lines inhibits cell growth (Uchida et al., 2014). The different tissues employed in those experiments could explain the controversial observations; hepatocellular cells are different from oral cancer cells, which derive from squamous epithelial tissue, as do CC cells.

An important observation was the upregulation of HOXA9 when HPV oncogenes were silenced. There is no previous evidence of HOXA9 regulation by HPV infection, or of HOX genes regulation by the E6 or E7 oncoproteins. More studies are needed in this regard, and our group is currently working on this. Although our study reinforces
the role of HOXA9 as tumor suppressor in the cervical epithelia, many questions remain concerning HOXA9 that have arisen from our and other observations. It is important to emphasize that the dual behavior of HOXA9 as an oncogene or tumor suppressor highlights the importance of tissue context in its function.

In summary, HOXA9 expression is downmodulated in some types of CC cells, and that could depend on the HPV type that it infects. HOXA9 downmodulation represents an advantage for HPV18-infected CC cells, which in that condition are more proliferative and have a high migration rate. The expression of HOXA9 is regulated by methylation and by the HPV oncogenes, and the restoration of HOXA9 induces lowers proliferation and the markers of epithelial-to-mesenchymal transition.

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval: “All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.”

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