HDAC7-mediated control of tumour microenvironment maintains proliferative and stemness competence of human mammary epithelial cells

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HDAC7 is a pleiotropic transcriptional coregulator that controls different cellular fates. Here, we demonstrate that in human mammary epithelial cells, HDAC7 sustains cell proliferation and favours a population of stem-like cells, by maintaining a proficient microenvironment. In particular, HDAC7 represses a repertoire of cytokines and other environmental factors, including elements of the insulin-like growth factor signalling pathway, IGFBP6 and IGFBP7. This HDAC7-regulated secretome signature predicts negative prognosis for luminal A breast cancers. ChIP-seq experiments revealed that HDAC7 binds locally to the genome, more frequently distal from the transcription start site. HDAC7 can colocalize with H3K27-acetylated domains and its deletion further increases H3K27ac at transcriptionally active regions. HDAC7 levels are increased in RAS-transformed cells, in which this protein was required not only for proliferation and cancer stem-like cell growth, but also for invasive features. We show that an important direct target of HDAC7 is IL24, which is sufficient to suppress the growth of cancer stem-like cells.

1. Introduction

Environmental adaptations require fine-tuning of gene transcription supervised by chromatin modifications. A repertoire of epigenetic regulators controls the milieu of the epigenetic marks that influence chromatin compaction or opening (Vineis et al., 2017). Among transcriptional repressors, class IIA HDACs are important regulators of genetic programmes, which orchestrate different cellular fates. This family of enzymes includes four members: HDAC4, HDAC5, HDAC7 and HDAC9, which are subjected to environmental regulated nuclear/cytoplasmic shuttling (Clocchiatti et al., 2011; Di Giorgio and Brancolini, 2016). In vertebrates, class IIA HDACs show neglectable lysine-deacetylase activity; however, through the recruitment of the N-CoR/SMRT/HDAC3 complex, they can coordinate and buffer histones acetylation (Desravines et al., 2017; Lahm et al., 2007). An extended amino-terminal region, devoted to the interaction with other corepressors and different transcription factors, grants selective genomic activities to these epigenetic regulators (Clocchiatti et al., 2011; Di Giorgio and Brancolini, 2016).

The ability of class IIA HDACs to act as platforms for the recruitment of different partners renders these coregulators highly tuneable in terms of genes modulated and consequent biological responses. This plasticity holds remarkably true for HDAC7. Genetic studies in mice have proved that Hdac7 controls vascular stability and remodelling (Chang et al., 2006), as well as...
B-cell and T-cell development (Azagra et al., 2016; Kasler and Verdin, 2007; Navarro et al., 2011).

Epigenetic plasticity is emerging as an important hallmark of cancer (Barneda-Zahonero and Parra, 2012; Feinberg et al., 2016; Flavahan et al., 2017; Koschmann et al., 2017). Up-regulation of HDAC7 expression in rodent and human cells can cooperate for the neoplastic transformation (Di Giorgio et al., 2013; Lei et al., 2017; Paluvai et al., 2018; Rad et al., 2010). In breast and ovary cancers, HDAC7 is highly expressed in cancer stem cells and it is involved in stem cell maintenance (Witt et al., 2017). Although there are evidences for a role of HDAC7 in the neoplastic transformation, the genetic and epigenetic changes under HDAC7 supervision are not fully elucidated. To achieve this goal, we knocked out HDAC7 in human mammary epithelial cells MCF10A, which offer the opportunity of studying proliferation, stemness and oncogenesis (Amin et al., 2016; Liu et al., 2013; Qu et al., 2015).

2. Materials and methods

2.1. Cell cultures and reagents

MCF10A cells were grown as previously described (Clocchiatti et al., 2015). HEK-293T, AMPHO and SKBR3 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Euroclone, Milan, Italy) supplemented with 10% FBS (Euroclone). Cells expressing the inducible form of HDAC7 were grown in complete F12/DMEM medium without phenol red (Sigma-Aldrich, St. Louis, MO, USA) and with 5% charcoal-stripped horse serum. 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) was used at 1 μM.

2.2. Three-dimensional morphogenetic and mammosphere assays

The 3D morphogenetic assay was conducted as previously described (Clocchiatti et al., 2015). Cells (3 × 10^4) were plated in a thick layer of ~1–2 mm of laminin-rich extracellular matrix (ECM; Cultrex-Sigma-Aldrich). Mammospheres were grown in mammosphere medium (MM) constituted by Ham's F12/C1 ([Sigma-Aldrich]). Mammospheres were counted. 1 × 10^5 cells were seeded on Ultra-Low Attachment multiwells (Corning, New York, NY, USA) and cultivated for 10 days. For BMP4 (Thermo Fisher, Waltham, MA, USA) and IL24 (Peprotech) treatments, MCF10A were pretreated for 2 days. Spheres over 50 μm of diameter were counted. Conditioned medium was obtained by growing MCF10A cells in complete MM for 2 days. Next, medium was collected, filtered and diluted 1 : 1 with fresh new MM and used for the experiments. Images were collected by using a Leica AF 6000LX microscope (Leica Microsystems, Mannheim, Germany). Area was measured with IMAGEJ software (NIH, Bethesda, MD, USA).

2.3. Generation of MCF10A/HDAC7−/− cells

CRISPR/Cas9 technology was used as previously described (Di Giorgio et al., 2017). Clones were screened by PCR and immunoblot. Sanger sequencing was applied for the final validation. gRNAs were designed using 'CRISPR design' available from http:// crispr.mit.edu/ gRNA exon 3: 5′ CACCGAGC GCTCGGTGGAGCCCATG 3′; gRNA exon 4: 5′ CA CGCCGATGCCCAGTGCAGG 3′; gRNA exon 5: 5′ CACCGGCTCAAGCAGAAGCTACGG 3′ cloned into the lentiviral plasmid pLentiV2 which expresses the Cas9 gene. CTRL cells were infected with the pLentiV2 lacking the guide.

2.4. Immunofluorescence and immunoblotting

Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. The secondary antibodies were Alexa Fluor 488-, 546- or 633-conjugated anti-mouse and anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR, USA). Actin was labelled with phalloidin-AF546 (Molecular Probes, Eugene, OR, USA). Nuclei were stained with Topro-3 (Life Technologies, Waltham, MA, USA) or Hoechst 33258 (Sigma-Aldrich).

Cell lysates after SDS/PAGE and immunoblotting were incubated with primary antibodies. Secondary antibodies were obtained from Sigma-Aldrich, and blots were developed with SuperSignal West Dura (Pierce, Waltham, MA, USA).

2.5. Antibodies

The primary antibodies used were as follows: MEF2D (BD Biosciences, San Jose, CA, USA); MEF2A (C-21 Santa Cruz Biotechnology, Dallas, TX, USA); p53 and pERK (Cell Signaling Technology, Leiden, the
Netherlands); p21, Actin, anti-BrdU, FLAG M2 and RACK-1 (Sigma-Aldrich); GFP and HDAC4 (Paroni et al., 2004); and HDAC5 (Clocchiatti et al., 2015). Anti-CD44-FITC (BD Biosciences) and CD24-APC (BioLegend, San Diego, CA, USA) were used with matched control antibodies, anti-mouse IgG1 FITC and IgG2a APC (Miltenyi Biotec, Bergisch Gladbach, Germany). HDAC7 antibodies were generated in rabbits by injecting recombinant histidine-tagged HDAC7 fragment aa 261–522. For anti-HDAC7 antibody purification, HDAC7 was fused to glutathione S-transferase and cross-linked to glutathione-Sepharose as described previously (Paroni et al., 2004).

2.6. Plasmid construction, transfection, retroviral and lentiviral infection, siRNA delivery

To generate pWZL-Hygro-HDAC7 WT-ER, an EcoRI fragment of HDAC7 WT was cloned into pWZL-Hygro-ER. Cells expressing HDAC7-ER, RAS-G12V, fragment of HDAC7 WT was cloned into pWZL-DBD transgenes were generated by retroviral infection as described previously (Cernottta et al., 2011).

2.7. RNA extraction and quantitative qRT/PCR

Cells were lysed using TRI Reagent (Molecular Research Center, Cincinnati, OH USA). About 1.0 µg of total RNA was retro-transcribed by using 100 units of M-MLV Reverse Transcriptase (Life Technologies). qRT/PCRs were performed using SYBR green technology (KAPA Biosystems, Wilmington, MA, USA). Data were analysed by comparative threshold cycle using HPRT as normalizer.

2.8. RNA expression array and data analysis

Aliquots of RNAs, purified using RNasy columns (Qiagen, Hilden, Germany), were amplified according to the specifications of the Illumina TotalPrep RNA Amplification Kit (Ambion, Waltham, MA, USA). Hybridization on Illumina whole-genome HumanHT-12 v 4.0 chip (Illumina, San Diego, CA, USA), scanning and background subtraction were done according to the manufacturer’s specification. Fold-change and P-values for each probe set were calculated using a moderated t-statistic in the limma package (Ritchie et al., 2015), with the variance estimate being adjusted by incorporating global variation measures for the complete set of probes on the array. P-values were then corrected for multiple hypothesis testing using the Benjamini and Hochberg methods. In order to select genes that are robust HDAC7 targets, we assumed that HDAC7+/+ and HDAC7−/−/HDAC7-ER samples could be considered as replicates, similarly to HDAC7−/− and HDAC7−/−/ER. Differentially expressed genes were selected for fold changes |> 1.5| and P values < 0.05. Gene set enrichment analysis (GSEA) and the MSigDB database http://software.broadinstitute.org/gsea/index.jsp (Liberzon et al., 2015; Subramanian et al., 2005) were used to investigate statistically significant functional associations.

2.9. Cell cycle FACS analysis, BrdU and transwell migration assays

For cyttofluorimetric analysis, cells were fixed with ethanol (O/N), treated with RNase A (AppliChem Lifescience, Darmstadt, Germany) and stained with 10 µg of propidium iodide (Sigma-Aldrich). For S-phase analysis, cells were grown for 3 h with 50 µM bromodeoxyuridine (BrdU; Sigma-Aldrich). After fixation, coverslips were treated with HCl and processed for immunofluorescence. Invasion assay was performed as previously described (Di Giorgio et al., 2017). As chemoattractant, complete DMEM/F12 with EGF was added in each lower chamber. DMEM/F12 without EGF was used to evaluate random invasion.

2.10. ChIP, library construction, ChIP-seq and NGS data analysis

ChIP was performed as previously described (Di Giorgio et al., 2017), with some modifications. In brief, 50 µg of chromatin was immunoprecipitated with 2 µg of anti-H3K27ac (Abcam; ab4729), 6 µg of anti-HDAC7 antibody or control IgG. After RNAseA treatment (Ambion) and de-crosslinking, DNA was purified with Zymo ChIP columns. Three independent experiments were pulled, and 5 ng of total DNA was used to prepare ChIP-seq libraries, according to TrueSeq ChIP Sample Preparation guide (Illumina). Libraries were sequenced on the Illumina HiSeq 2000 sequencer. The quality of sequencing reads was evaluated using FastQC. Sequencing reads from ChIP-seq experiments were aligned to the NCBI GRCh38 human reference with bowtie 2 (Langmead and Salzberg, 2012). Peak calling was performed against input sequences using the homer software (Heinz et al., 2010). Peak heatmaps, gene annotations, Venn diagrams and bar plots representing the peak localization in genomic elements/distance from transcription start site (TSS) were obtained using the ChIPseeker R/Bioconductor package (Yu et al., 2015).
2.11. Statistics

Results were expressed as means ± standard deviations for at least three independent experiments. Statistical analysis was performed using Student’s t test with the level of significance set at \( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.005 \). Data from 3D acinar area and mammosphere formation assays were analysed using the one-way ANOVA (and nonparametric) test (PRISM GraphPad software; GraphPad software, La Jolla, CA, USA).

3. Results

3.1. HDAC7 influences proliferation of mammary epithelial cells

The CRISPR/Cas9 technology was used to generate HDAC7\(^{-/-}\) MCF10A mammary epithelial cells. We characterized in parallel two different clones generated by two different pairs of gRNAs (Fig. S1A). HDAC7 abrogation does not trigger compensatory feedbacks at the levels of other class IIa HDACs and MEF2 family members expressed in MCF10A cells (Fig. 1A). HDAC9, MEF2B and MEF2C are expressed at very low levels when grown in suspension (Amin et al., 2012). Since a recent study claimed a role of HDAC7 in breast cancer stem cells (Witt et al., 2017), we compared sphere generation by HDAC7\(^{+/+}\) and HDAC7\(^{-/-}\) cells. As previously reported (Amin et al., 2016; Clément et al., 2017; Qu et al., 2015), MCF10A cells can form spheres with low efficiency (Fig. 2A). Of note, sphere formation was almost suppressed in HDAC7\(^{-/-}\) cells and HDAC7\(^{-/-}\) cells expressing the ER alone (Fig. 1G–I). The rescue of the proliferative deficit was similarly observed after re-expression of the nuclear resident HDAC7 protein (Fig. S2). In summary, these data demonstrate that HDAC7, when present in the nucleus, sustains MCF10A cell proliferation, possibly through the control of CDKN1A.

3.2. HDAC7 and mammosphere formation

MCF10A cells can form spheres with low efficiency when grown in suspension (Amin et al., 2016; Clément et al., 2017; Qu et al., 2015). This \textit{in vitro} assay is commonly used to measure the presence of rare stem/progenitor-like cells (Shaw et al., 2012). To unambiguously prove the effect on cell proliferation of HDAC7, an inducible version of HDAC7, fused to ER (oestrogen receptor), was re-introduced in HDAC7\(^{-/-}\) cells. The same cells expressing the ER alone were also generated. Treatment with 4-OHT stabilized the expression of the protein (Fig. 1F). Inhibition of nuclear export by leptomycin B treatment proved that, similarly to the endogenous HDAC7, HDAC7-ER undergoes nuclear/cytoplasmic shuttling (Fig. S1B). The increase in CDKN1A/p21 levels (Fig. 1G,H) and the proliferative defects of HDAC7\(^{-/-}\) cells (Fig. 1I) were completely rescued by the re-expression of HDAC7-ER, but not by the expression of the ER alone (Fig. 1G–I). The rescue of the proliferative deficit was similarly observed after re-expression of the nuclear resident HDAC7 protein (Fig. S2). In summary, these data demonstrate that HDAC7, when present in the nucleus, sustains MCF10A cell proliferation, possibly through the control of CDKN1A.
the rarely generated spheres were reduced in size (Fig. 2A and Fig. S3A,B). The combination of the surface markers CD44+/CD24low was used to distinguish stem-like cells (Amin et al., 2016; Clément et al., 2017; Shaw et al., 2012). We did not observe such a reduction of this population in HDAC7−/− cells, to justify the failure in the mammosphere assay (Fig. 2B,C). Serial passages of the mammospheres were performed...
to assess their self-renewal capacity. At the third generation, HDAC7−/− cells almost exhausted the stem-like features (Fig. 2D).

The behaviour of stem cells can be influenced by different environmental factors (Shaw et al., 2012). Particularly, previous studies have shown that BMP4 can increase the number of mammospheres generated by MCF10A cells (Clément et al., 2017). When both HDAC7+/+ and HDAC7−/− cells were treated with BMP4, the number of spheres was augmented.

Fig. 2. HDAC7 contributes to mammosphere generation in MCF10A cells. (A) Scatter dot plot illustrating the number of mammospheres generated by MCF10A/HDAC7+/+ or HDAC7−/− cells after 10 days in culture. n = 9. We marked with ***P(Dunn) < 0.005. (B) Representative FACS analysis of CD44 and CD24 (upper panel) or matched control antibodies (lower panel) in MCF10A/HDAC7+/+ or HDAC7−/− cells. (C) Quantitative analysis of CD44+/CD24low cell population as performed by FACS in HDAC7+/+ or HDAC7−/− MCF10A cells. Data are presented as mean ± SD (n = 3). We marked with *P(Student) < 0.05. (D) Formation and serial passages of mammospheres as generated by HDAC7+/+ and HDAC7−/− MCF10A cells. Data are presented as mean ± SD (n = 3). We marked with ***P(Student) < 0.005. (E) Scatter plots illustrating the number of mammospheres generated by MCF10A HDAC7+/+, HDAC7−/−, HDAC7−/−ER and HDAC7−/−HDAC7-ER cells, as indicated. For experiments with BMP4, cells were pretreated for 2 days with 15 ng·mL−1 of BMP4. n = 9. We marked with ***P(Dunn) < 0.005.
However, fewer spheres were again generated by \( \text{HDAC7}^{-/-} \) cells (Fig. 2E). Importantly, HDAC7 re-expression fully recovered the defect of KO cells in mammosphere formation (Fig. 2E and Fig. S3A,B).

### 3.3. HDAC7 regulates a repertoire of genes involved in extracellular microenvironment reprogramming

In order to map the genetic programmes supervised by HDAC7, we compared the transcriptomes of \( \text{HDAC7}^{+/+} \) and \( \text{HDAC7}^{-/-} \) cells. To prove that fluctuations in gene expression were a specific consequence of HDAC7 inactivation, we included the gene expression profiles of MCF10A/\( \text{HDAC7}^{+/+} \)/HDAC7-ER and its control MCF10A/\( \text{HDAC7}^{-/-} \)/ER, grown in the presence of 4-OHT. We found 476 genes (\(|fc| > 1.5; \ P < 0.05\)) under HDAC7 influence, of which 272 were up-regulated and 204 were down-modulated (Fig. 3A, Table S1). Among these 476 differentially expressed genes, \( \text{IL24, CCL20, and FBXO32} \) resulted the strongest up-regulation, whereas \( \text{SPRR3, SPRR1A} \) and \( \text{KRTDAP} \) showed the strongest down-modulation (Fig. 3B). Since HDAC7 is a transcriptional repressor, we focused the analysis on genes up-regulated in \( \text{HDAC7}^{-/-} \) cells. The GSEA and the Molecular Signatures Database (MSigDB) analysis showed the up-regulation of genes involved in the inflammatory response (interferon-\( \alpha \) and \( \gamma \) responses) and the xenobiotic response in \( \text{HDAC7}^{-/-} \) cells (Fig. 3C). ECM, immune system and cytokine signalling resulted in the first three enriched MSigDB curated gene set categories (Fig. 3D). Finally, the top-ranking GO biological processes were the defence response, the response to external stimulus and the negative regulation of cell proliferation (Fig. 3E). Similarly, the top GO term analysis revealed the enrichment of the GO term cell adhesion, type I interferon signalling pathway and signal peptide in \( \text{HDAC7}^{-/-} \) cells (Fig. 3F). Overall, many genes repressed by HDAC7 modulate the extracellular microenvironment, including the inflammatory response and cell adhesion.

### 3.4. HDAC7 depletion affects a pro-stemness programme triggered by BMP4

To understand the influence of HDAC7 on the ability of BMP4 to promote mammosphere growth, we compared the gene expression profiles of BMP4-treated \( \text{HDAC7}^{+/+} \) and \( \text{HDAC7}^{-/-} \) cells. BMP4 promoted the up-regulation of 182 and 184 genes, respectively, in \( \text{HDAC7}^{+/+} \) and in \( \text{HDAC7}^{-/-} \) cells (\(|fc| > 2.0; \ P < 0.05\)) (Fig. S4A and Table S2). Seventy-five genes were commonly up-regulated in the two cell lines. As expected, BMP4 triggered the TGF-\( \beta \) signalling, in both WT and KO cells (Fig. S4B,C). This result implies that the genetic programmes activated by BMP4 are not overtly compromised by the absence of HDAC7, even though some minor changes can be appreciated (Fig. S4B,C). A stronger quantitative difference regards genes down-regulated by BMP4. One hundred and seventy-three genes were repressed in the presence of HDAC7 and only 93 in its absence (\(|fc > 2.0; \ P < 0.05\)) (Fig. S4D and Table S3). The epithelial–mesenchymal transition emerged as the genetic programme repressed by BMP4 in both \( \text{HDAC7}^{+/+} \) and \( \text{HDAC7}^{-/-} \) cells (GSEA on chemical and genetic perturbations). When GSEA was done with other gene sets, few overlapping results were found between \( \text{HDAC7}^{+/+} \) and \( \text{HDAC7}^{-/-} \) cells (Fig. S4E,F).

It is reasonable to hypothesize that the reduced pro-stem activity of BMP4 in \( \text{HDAC7}^{-/-} \) cells (Fig. 2E) could depend on the deficit in repressing few genes important for stemness. These genes should be repressed by BMP4 in WT cells but not in KO cells. Importantly, they should not be repressed in untreated \( \text{HDAC7}^{-/-} \) cells. Hence, we selected all genes repressed by BMP4 in \( \text{HDAC7}^{+/+} \) (\(|fc > 2.0, \ n = 173\)). Next, their expression was compared with genes repressed after HDAC7 knock-out and genes repressed by BMP4 treatment in \( \text{HDAC7}^{-/-} \) cells. 31 genes were repressed by BMP4 in a HDAC7-dependent manner (Fig. 4A). GSEA was performed on this 31-gene signature and the top curated gene set identified was the ‘mammary stem cells down-regulated genes’ (Fig. 4B, C). qRT/PCR analysis was performed for \( \text{MUC1, ELF3} \) and \( \text{ISG20} \). All these three genes were up-regulated in \( \text{HDAC7}^{-/-} \) cells, down-regulated in response to BMP4, and the BMP4-dependent suppression was inefficient in \( \text{HDAC7}^{-/-} \) cells (Fig. 4D).

### 3.5. Identification of a HDAC7-regulated secretome signature that sustains stemness and predicts a negative prognosis in luminal A breast cancers

The transcriptomic analysis revealed that HDAC7 represses the expression of 40 genes encoding for secreted factors that we named ‘HDAC7-secretome signature’ (Table S4). These include: (a) chemokine ligands \( \text{CCL5, CCL20, CCL24} \), (b) \( \text{ISG15} \), which acts also extracellularly to favour IFN-\( \gamma \) secretion (Zhang et al., 2015), (c) interleukins \( \text{IL1B} \) and \( \text{IL24} \), and (d) elements of the insulin-like growth factor (IGF) signalling (\( \text{IGFBP6, IGF87, IGFL2} \)). The new HDAC7

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targets such as the inhibitors of IGF signalling (IGFBP6 and IGFBP7), interleukins IL1B and IL24 and the interferon-inducible gene ISG15 were validated by qRT/PCR (Fig. 5A). We also included ERRFI1, which encodes for a negative regulator of the RTK signalling (Segatto et al., 2011), and the E3 ligase FBXO32/Mafbx/Atrogin1, involved in muscular atrophy (Bodine and Baehr, 2014). All tested genes were up-regulated in HDAC7+/− cells and repressed upon HDAC7 re-activation (Fig. 5A). GSEA proved that a role of HDAC7 in the regulation of the secretome is not restricted to MCF10A cells. Part of this signature is up-regulated also in human endothelial progenitor cells, after HDAC7 silencing. Importantly, also in these cells IL24 is the most responsive gene (Fig. S5A, B; Wei et al., 2018).

The influence of HDAC7 on the microenvironment could have important implications in vivo. When
luminal A breast cancer patients were stratified according to the expression levels of the HDAC7-secretome signature (Curtis et al., 2012), patients with high levels of the signature showed a better prognosis (Fig. 5B). This effect could be due to an impairment on cancer stem properties caused by the activation of this signature. To prove this hypothesis, we compared the conditioned medium taken from HDAC7+/+ and HDAC7−/− cells for the capability of sustaining mammosphere generation. Conditioned medium from HDAC7+/+ cells strongly increased the number of spheres generated, whereas the medium from HDAC7−/− cells did not provide advantages (Fig. 5C).

HDAC7 indirectly controls gene expression by forming complexes with TF. Important partners of HDAC7 are the MEF2 family members (Di Giorgio et al., 2018). To understand whether genes under HDAC7 influence are MEF2 targets, we expressed an inducible hyperactive version of MEF2 in MCF10A cells (Clocchiatti et al., 2015). As a control, a MEF2 deleted in the DNA binding domain was used. qRT/PCR analysis indicated that FBXO32, ERRFI1 and IL24 are under MEF2 regulation. By contrast, IGFBP6 and IGFBP7 are not MEF2 targets (Fig. 5D).

3.6. Mapping HDAC7 activities at the genomic level

The above study indicates that HDAC7 can influence the expression of genes also independently from MEF2 TFs. These genes could be direct or indirect targets of HDAC7. As a first step to clarify the genomic functions of HDAC7, we performed chromatin immunoprecipitation sequencing (ChIP-seq) in HDAC7+/+ and HDAC7−/− cells. We also compared the distribution of the histone H3 acetylated on lysine 27...
(H3K27ac), a marker of transcriptionally active promoters and enhancers.

Overall, H3K27ac-enriched regions were increased in the absence of HDAC7 (139,328 peaks compared to 95,419 in HDAC7+/+ cells). The analysis of the genomic distributions of these marks revealed an increase in H3K27ac at introns and distal intergenic regions in HDAC7−/− cells (Fig. 6A). We found 5072 enriched peaks for HDAC7 in WT cells. Nine hundred and eighty-nine and 1380 of them colocalized (±1 kb interval) with H3K27ac peaks, respectively, in HDAC7+/+ and HDAC7−/− cells. This evidence suggests that de novo H3K27ac can be elicited after HDAC7 suppression.

Comparison of the H3K27ac peak distribution with respect to the TSS in HDAC7+/+ and HDAC7−/− cells confirmed that HDAC7 does not play a global activity, being the pattern of H3K27ac distribution almost superimposable between the two cell lines (Fig. 6B and Fig. S6). Despite this, HDAC7 could...
operate on selected TSS. To verify this hypothesis, we focused the analysis on the H3K27ac status at the TSS of the 272 genes up-regulated in HDAC7−/+ cells. We excluded transcripts with undefined functional annotations, thus resulting in 155 genes.

In HDAC7−/+ cells, the H3K27ac status at the ±3 kb regions around the TSSs evidenced a robust increase for the vast majority of the 155 genes. The H3K27ac increase was more frequently observed downstream from the TSS (Fig. 6C). IL24, OPN3 and IRF6 were the top genes for H3K27ac rise, within the ±3 kb region. The regions where these three genes displayed a strong increase of H3K27ac (around ±1 kb from the TSS), were, at the same time, also enriched for HDAC7 binding peaks (IL24 −1580; OPN3 −825; IRF6 −962 from the TSS). Only 8 loci, among the 155 analysed, showed enriched peaks for HDAC7 in regions close to the TSS. It is possible that the deacetylase binds other regulative elements located more distally, with respect to the TSS (Azagra et al., 2016). Indeed, the genomic distribution of HDAC7 peaks evinced that approx. 70% of HDAC7 binding occurred in intergenic and intronic regions, while only a reduced percentage (approximately 26%, including the first intron) in regions near to the TSS and only 13% at promoters (Fig. 6D). Out of the 2118 HDAC7 peaks located in the intergenic regions, 204 were acetylated in WT cells (±1 kb) and 272 in KO cells. In summary, the vast majority of intergenic regions bound by HDAC7 seem to be transcriptionally inactive and only a fraction (approx. 9.6%) are in open chromatin. Removal of HDAC7 increases these H3K27ac intergenic regions up to 12.8%. It is possible that within these regions, some distal regulative elements, such as enhancers, could be found.

When the same analysis was performed on the 155 genes up-regulated in HDAC7−/+ cells, the percentage of promoter sequences enriched for HDAC7 binding raised up to more than 20%. Based on these evidences, we analysed H3K27ac and HDAC7 peak distributions in a wider region (−30 kb/30 kb) from the TSS of the 155 genes. In this interval, 21 out of 155 loci showed at least an enriched peak for HDAC7 (Fig. 6E, Table S5). In some cases, HDAC7 peaks colocalized with regions where H3K27ac was increased in a HDAC7-dependent manner. In other regions, this colocalization was not observed. In 50% of cases, at least one putative MEF2 binding site was present within HDAC7-enriched peaks associated with these 21 genes (P-value ≤ 0.05).

As representative examples of the ChIP-seq data, the IL24 and CDKN1A loci are shown in Fig. 6F,G. These genes emerged as two HDAC7 direct targets, which show the colocalization of HDAC7 and H3K27ac peaks.

### 3.7. Roles of HDAC7 in the transformation of human breast epithelial cells

To explore the contribution of HDAC7 to breast cell transformation and to CSC features, we introduced the RAS oncogene in HDAC7−/+ and in HDAC7+/+ cells. RAS elicits a transformed phenotype, characterized by invasive and anchorage-independent growth (Liu et al., 2013; Moon et al., 2000). RAS was similarly expressed in the two cell lines, and ERK phosphorylation was dramatically augmented, independently from HDAC7 (Fig. 7A). Interestingly, HDAC7 levels were increased in cells overexpressing RAS (Fig. 7A). Comparative analysis of HDAC7 sub-cellular localization revealed that in MCF10A cells, the deacetylase shows a uniform nuclear/cyttoplasmic localization. In RAS-transformed cells, HDAC7 localization is more heterogeneous. Some cells show higher nuclear staining while others a stronger cytosolic signal (Fig. S7).

Cell proliferation was augmented by RAS in both HDAC7−/+ and HDAC7+/+ cells. However, in the absence of HDAC7, RAS-transformed cells retain the S-phase deficit previously described (Figs 1B and 7B). When grown in 3D conditions, RAS-transformed cells adopt an invasive stellate growth pattern (Giunciuglio et al., 1995). We observed that in 3D conditions, MCF10A/RAS cells can generate acini with a normal spheroid morphology, as well as stellate acini, which show features of invasive growth (Fig. 7C,D). Elimination of HDAC7 reduces the invasive growth in 3D culture of MCF10A/RAS cells (Fig. 7E). To confirm the role of HDAC7 in sustaining an invasive behaviour, MCF10A/RAS, HDAC7+/+ or HDAC7−/+ cells were plated on Matrigel-coated transwell filters to evaluate their migratory properties. As expected, RAS enhanced invasion in Matrigel, but, in the absence of HDAC7, this invasive phenotype was reduced (Fig. 7F).

When RAS-transformed cells were analysed for the surface markers CD44 and CD24, the cytofluorimetric analysis indicated a dramatic increase in the stem-like population (Fig. 7G,H). The increased stem features of MCF10A/RAS cells were confirmed by the compelling increase in the numbers of mammospheres generated, compared to untransformed cells (Fig. 7I). Importantly, the absence of HDAC7 dramatically reduced the mammosphere generation also in MCF10A/RAS cells.
This reduction, in conjunction with the absence of effects on the CSC population, as determined by CD44+/CD24low markers (Fig. 7H), suggests that the impact of HDAC7 on the microenvironment could be again a key aspect to sustain the growth of CSCs.
This consideration prompted us to evaluate whether genes, encoding for secreted factors and up-regulated in HDAC7 KO cells, were regulated also in MCF10A/RAS cells. Secreted factors, specifically up-regulated in HDAC7−/− cells also in the presence of RAS, are strong candidates as suppressors of mammosphere growth. ISG15, IGFBP6, IGFBP7 and IL24 expression levels were evaluated (Fig. 7J). IL8/CXCL8, a well-known RAS target that enhances cell proliferation, was included as control (Sparmann and Bar-Sagi, 2004; Zheng et al., 2015). IGFBP7 was strongly repressed by RAS. IGFBP6, instead, was up-regulated by RAS through a HDAC7-dependent mechanism. ISG15 was modestly up-regulated by RAS. As expected, IL8 was strongly up-regulated by RAS and this up-regulation was even stronger in the absence of HDAC7. Finally, IL1B and IL24 were strongly up-regulated in a HDAC7-dependent and RAS-independent manner. In summary, these results indicate that part of the HDAC7-regulated secretome can be influenced during cellular transformation, also independently from HDAC7. The deacetylase maintains a strong repressive influence versus IL24, IL1B and IL8.

Since IL24 is an important HDAC7 target and since its antiproliferative activities are well documented, its repression could be critical to favour a proficient microenvironment for stem-like cells growth. To verify this hypothesis, we treated MCF10A/RAS cells with IL24. We confirmed previous studies showing that IL24 can trigger a certain amount of apoptosis in cancer cells (Fig. 7K). To perform the mammosphere assay, only trypan blue-negative healthy cells were used. Mammosphere generation by MCF10A/RAS cells was dramatically impaired by IL24 (Fig. 7L). The quantitative analysis proved the inhibitory effect of IL24 on mammosphere generation and growth, when used in the pre-treatment, as well as when its presence was maintained throughout the assay (Fig. 7M).

4. Discussion

The contribution of class IIa HDACs to cell proliferation and transformation is underestimated. There are some evidences about the transforming potential of HDAC7 and its activity as oncogene (Di Giorgio et al., 2013; Lei et al., 2017; Paluvai et al., 2018; Peixoto et al., 2016). Here, we have proved that, in human mammary epithelial cells, HDAC7 influences multiple aspects of the transformation process including proliferation, invasion and stemness. The transcriptomic analysis revealed that a consistent pool of genes repressed by HDAC7 encodes for secreted and plasma membrane proteins. In particular, the expression of inflammatory and antiproliferative cytokines, mediators of the immune response and negative regulators of IGF signalling were repressed by HDAC7. This specific milieu of factors is particularly critical to sustain the growth of stem-like cells. Importantly, repression of this microenvironmental signature correlates with the aggressiveness of luminal A breast cancers. Not surprisingly, since HDAC7 is regulated at multiple levels, including protein stability and nuclear-cytoplasmic shuttling (Di Giorgio and Brancolini, 2016), we have not found a correlation between its mRNA levels and luminal A tumour aggressiveness.

A role of HDAC7 in the regulation of the microenvironment was previously suggested (Peixoto et al., 2016). Here, we have proved that the microenvironment is influenced by HDAC7 and it is involved in the sustainment of the stem-like population. Among microenvironmental genes influenced by HDAC7, we found IGFBP6 and IGFBP7, proteins that can buffer IGF signalling (Bach, 2015; Evdokimova et al., 2012). IGFBP7 expression was strongly repressed also by RAS, regardless of HDAC7. Hence, IGFBP7 could represent a common target of different signalling pathways to sustain stem cell features. In this respect, a
role of IGFBP7 as inhibitor of the expansion and aggressiveness of tumour stem-like cells was recently proved in vivo (Cao et al., 2017).

HDAC7 represses also some chemokines and cytokines. In the case of IL24, the involvement of MEF2s is plausible. Abrogation of IL24 repression, following HDAC7 deletion, seems to be a critical antiproliferative signal. In fact, addition of this cytokine dramatically inhibited mammosphere generation by MCF10A/RAS cells.

\( \text{IL24/MDA-7, originally identified as a gene whose expression is induced during melanoma differentiation, inhibits tumour growth in different contexts (Bhutia et al., 2013; Menezes et al., 2014). IL24 belongs to the IL10 gene family and exerts different antitumoural activities such as induction of apoptosis, suppression of invasion/metastasis and anti-angiogenic effects (Menezes et al., 2014). Different mechanisms have been proposed to explain its pro-apoptotic activity including intracellular and environmental signalling (Bhutia et al., 2013; Dash et al., 2014; Lebedeva et al., 2002; Menezes et al., 2014).\}

HDAC7 binds the promoter of IL24 at \(-1580\) and \(-6574\) from the TSS and influences the H3K27 acetylation status in a wider genomic region. Genomic analysis has defined that HDAC7 more frequently binds intergenic regions or regions distal from TSS. Only in a small percentage of cases, the binding of HDAC7 was observed close to the TSS. Not surprisingly for a repressor, HDAC7 can be found in regions marked by low H3K27ac levels, which should indicate the maturation of a repressed chromatin status. However, HDAC7 was detected also in transcriptionally active domains, characterized by high amounts of H3K27ac. Here, it actively contributes to buffer the acetylation levels.

The impact of HDAC7 on cytokine production is not limited to IL24. \( \text{IL1B was identified among genes up-regulated in HDAC7}^{-/-}\) cells, and \( \text{IL8 was strongly up-regulated in RAS-transformed HDAC7}^{-/-}\) cells. We observed that also \( \text{IL32 is repressed by HDAC7} \) (data not show). Since these cytokines can exert opposite effects on proliferation and stemness and operate also as paracrine factors, further studies will be necessary to clarify the influence of HDAC7 on the microenvironment.

In addition to the microenvironment, also cellular factors repressed by HDAC7 contribute to growth/survival of stem-like cells. In fact, addition of BMP4 was not sufficient to fully recover the defect of HDAC7\(^{-/-}\) cells. Among genes, possibly involved in this response, the panel of 7 genes repressed by BMP4 in a HDAC7-dependent manner and consistently down-regulated in mammary stem cells (Lim et al., 2010) are good candidates that should be tested in the future.

**5. Conclusions**

In conclusion, our study adds another piece of evidence about the contributions of HDAC7 to multiple
proliferative options, including the regulation of stem-like cells. Overall, our results encourage further efforts to discover and evaluate HDAC7-specific inhibitors (Di Giorgio et al., 2015) in a therapeutic perspective.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

CB designed research; RP, ED, EDG and CB analysed data; VC, EDG and MM performed research; and VC, EDG, ED, CB wrote the paper.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Characterization of MCF10A HDAC7<sup>−/−</sup> cells.

**Fig. S2.** The nuclear resident version of HDAC7 rescues the proliferative defect of HDAC7<sup>−/−</sup> cells.

**Fig. S3.** HDAC7 and mammospheres generation potential.

**Fig. S4.** The transcriptomic response to BMP4 treatment.

**Fig. S5.** HDAC7 and the secretome.

**Fig. S6.** Distance from TSS of H3K27ac peaks in HDAC7<sup>+/+</sup> and HDAC7<sup>−/−</sup> MCF10A cells.

**Fig. S7.** Subcellular localization of HDAC7 in MCF10A transformed or not with RAS.

**Table S1.** List of the 476 genes regulated by HDAC7.

**Table S2.** List of the genes regulated by BMP4 in HDAC7<sup>+/+</sup> cells.

**Table S3.** List of the genes regulated by BMP4 in HDAC7<sup>−/−</sup> cells.

**Table S4.** List of the 40 genes characterizing the signature of HDAC7-repressed secreted factors.

**Table S5.** HDAC7wt enriched peaks associated with microarray up-regulated genes.