The MEF2–HDAC axis controls proliferation of mammary epithelial cells and acini formation in vitro

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ABSTRACT

The myocyte enhancer factor 2 and histone deacetylase (MEF2–HDAC) axis is a master regulator of different developmental programs and adaptive responses in adults. In this paper, we have investigated the contribution of the axis to the regulation of epithelial morphogenesis, using 3D organotypic cultures of MCF10A cells as a model. We have demonstrated that MEF2 transcriptional activity is upregulated during acini formation, which coincides with exit from the proliferative phase. Upregulation of the transcription of MEF2 proteins is coupled to downregulation of HDAC7, which occurs independently from changes in mRNA levels, and proteasome- or autophagy-mediated degradation. During acini formation, the MEF2–HDAC axis contributes to the promotion of cell cycle exit, through the engagement of the CDK inhibitor CDKN1A. Only in proliferating cells can HDAC7 bind to the first intron of the CDKN1A gene, a region characterized by epigenetic markers of active promoters and enhancers. In cells transformed by the oncogene HER2 (ERBB2), acini morphogenesis is altered, MEF2 transcription is repressed and HDAC7 is continuously expressed. Importantly, reactivation of MEF2 transcriptional activity in these cells, through the use of a HER2 inhibitor or by enhancing MEF2 function, corrected the proliferative defect and re-established normal acini morphogenesis.

KEY WORDS: HDAC7, HDAC4, MEF2A, MEF2D, p21, CDKN1A, Cell cycle, Morphogenesis, Apoptosis, HDAC5, Breast cancer, HER2, ERBB2, Laptatinib, 3D culture, H3K27

INTRODUCTION

Mammary morphogenesis is characterized by the presence of several different instructive signals, which provide the correct gene expression network to generate the proper glandular architecture. These structures transit from a disorganized state to an ordered epithelial organization that modulates polarity, proliferation and luminal cell clearance (Debnath et al., 2002; Lewandowski and Piwnica-Worms, 2014). Mammary epithelial MCF10A cells that have been cultured on a reconstituted extracellular matrix [three dimensional (3D) culture] undergo a morphogenetic process that resembles events found in vivo to generate acinar-like spheroids (Debnath et al., 2002, 2003; Streuli et al., 1991). Initially, each single cell proliferates to generate a filled spheroid. During this morphogenetic process, cells of the outer layer enter G0 (proliferation arrest) and polarize (Debnath et al., 2002; Weaver et al., 2002; Whyte et al., 2010). The outer layer of cells that contact the extracellular matrix (ECM) survive, whereas the inner core of cells, lacking basement membrane contacts, die through both apoptotic and non-apoptotic processes (Debnath et al., 2002; Mills et al., 2004; Wang et al., 2003). Integrin signaling plays a key role at different steps of this morphogenetic process (Lee and Streuli, 2014; Reginato et al., 2003), and the absence of integrin-mediated pro-survival signals (anoikis) promotes the expression and activity of pro-apoptotic factors. Death of the inner cells is responsible for the formation of hollow lumen-containing acini.

Transcriptional nodes that are responsible for the genetic reprogramming that controls this complex morphogenetic process are not completely defined. The myocyte enhancer factor 2 (MEF2) family of transcription factors, including the MEF2A, MEF2B, MEF2C and MEF2D isoforms, plays important functions in tissue development and homeostasis (Potthoff and Olson, 2007). Genetic studies in mice have proved the essential contribution of Mef2a to the regulation of the cardiovascular system (Naya et al., 2002). Likewise, defects in Mef2c impact on heart development, causing mice to die at embryonic day (E)9.5 (Lin et al., 1998, 1997). By contrast, mice with homozygous mutations in Mef2d are viable, probably because of overlapping expression patterns with other MEF2 members (Arnold et al., 2007). Conditional cell-lineage-specific deletions of MEF2s have shown that there are additional roles for these transcription factors during B-cell development (Debnath et al., 2013) and bone homeostasis (Collette et al., 2012; Kramer et al., 2012).

MEF2s influence the expression of numerous genes, depending on and in cooperation with other transcription factors (Potthoff and Olson, 2007). They can also operate as transcriptional repressors, when in complex with class-IIa histone deacetylases (HDACs) (Gregoire et al., 2006; Lu et al., 2000). HDAC4, HDAC5, HDAC7 and HDAC9 belong to the class-IIa subfamily, and they are distinguished by (i) the presence of an N-terminal regulatory region that is involved in binding to transcription factors and additional co-repressors, (ii) a C-terminal region that includes the catalytic domain, which in vertebrates exhibits an impaired Lys-deacetylase activity and (iii) nuclear-cytoplasmic shuttling in a signal-responsive fashion (Clocchiatti et al., 2013a, 2011; Yang and Seto, 2008). The intimate relationships between MEF2s and these repressors support the concept of a MEF2–HDAC axis.

Deregulated MEF2 transcriptional activities can impact on tumorigenesis, and recent results have demonstrated alterations in MEF2 levels and transcription in breast tumors (Clocchiatti et al., 2013b; Ma et al., 2014; Schuetz et al., 2006). In estrogen receptor (ER)-positive tumors, repression of putative MEF2-target genes...
correlates with aggressiveness, and high class-IIa HDAC expression is associated with reduced survival (Clocchiatti et al., 2013b). By contrast, in recurrent ER-positive mammary cancers, expression of MEF2s correlates with NOTCH1 protein levels (Pallavi et al., 2012). Although some reports point to a contribution of MEF2-dependent transcription to mammary gland neoplastic pathogenesis, data on the role of the MEF2–HDAC axis during normal gland development and homeostasis are lacking, and only little information is available on the role of the axis in epithelial cells (Ishikawa et al., 2010). Based on this evidence, we hypothesized that the MEF2–HDAC axis plays a role in the regulation of breast epithelial cell proliferation and/or differentiation. In this work, we have investigated the contribution of the axis to acini morphogenesis using an experimental model of MCF10A cells cultured in three dimensions.

RESULTS

Regulation of the MEF2–HDAC axis during acini morphogenesis

The first indication that the MEF2–HDAC axis contributes to mammary breast epithelial homeostasis resulted from the association of MEF2D expression with a better overall survival of individuals with breast cancer (Fig. 1A). Furthermore, MEF2D expression was significantly higher in normal samples compared to that in aggressive grade three cancers (Fig. 1B). Because high grading is associated with compromised glandular architecture and differentiation (Elston and Ellis, 2002), we decided to investigate the contribution of the MEF2–HDAC axis to mammary epithelial morphogenesis and maintenance. To gain insight into which members are expressed in this context, we compared expressed sequence tag (EST) profiles between skeletal muscle and breast tissue for class-IIa HDACs and MEF2A, MEF2C and MEF2D (Fig. 1C). As expected, in skeletal muscle, MEF2C, HDAC4 and HDAC5 were the most expressed members of each family. By contrast, in breast tissue, MEF2A, MEF2D and HDAC7 were the most expressed isoforms. Furthermore, Gene Set Enrichment Analysis (GSEA), using a signature of putative MEF2-target genes, suggested that MEF2-dependent transcription was positively modulated during MCF10A acinar morphogenesis (Fig. 1D). Hence, we selected MCF10A cells to investigate the role of the MEF2–HDAC axis during epithelial morphogenesis. When plated onto laminin-rich ECM (for 3D culture), MCF10A cells transited from an initial proliferative disorganized state (Fig. 1E, day 1), to a morphogenesis. When plated onto laminin-rich ECM (for 3D culture), MCF10A cells transited from an initial proliferative disorganized state (Fig. 1E, day 4), evidenced by the presence of several mitotic figures per acinus (Fig. 1F) and by the random orientation of the Golgi, to a morphogenesis but elicits different compensatory mechanisms

MEF2D downregulation does not affect acinar morphogenesis but elicits different compensatory mechanisms

MEF2D transcriptional activities could play roles during acinar morphogenesis. To comprehend this role, we silenced MEF2D expression by using lentiviral infection with small hairpin (sh) RNAs. We selected MEF2D because (i) MEF2D is the most expressed isoform in breast tissue (Fig. 1A) and (ii) its expression is upregulated during morphogenesis (Fig. 2B,D). Despite an evident downregulation of MEF2D at the mRNA and protein levels, MCF10A cells expressing two different shRNAs against MEF2D (Fig. S1A,B) did not show any evidence that the expression of the MEF2-target genes had been negatively altered. Instead, unexpectedly, ENO1 mRNA levels were augmented (Fig. S1B). In cells with downregulated MEF2D, acinar morphogenesis appeared to be normal, as proved by scoring the luminal filling per structure and Golgi orientation (Fig. S1C). We also evaluated whether the absence of MEF2D could influence the initial proliferation phase by measuring the acinar size. Here, again MEF2D seemed to be dispensable (Fig. S1D). The absence of evident phenotypes in cells that had been silenced for MEF2D could be the consequence of redundancy and/or of compensatory
Fig. 1. See next page for legend.
cell death (data not shown), thus indicating that MEF2 perturbations are principally linked to the proliferative phase of the morphogenetic process. Overall, these data suggest that, in order to proceed with the normal morphogenetic process in the mammary epithelial cells, MEF2 transcriptional activity must be restrained during the initial proliferative phase.

**Sustained HDAC7 activity promotes cell proliferation and affects acinar morphogenesis**

The demonstration of a role for MEF2-dependent transcription in limiting the proliferation of mammary epithelial cells indicates that the downregulation of HDAC7, which is observed during the morphogenetic process, could be instrumental to drive the exit from the cell cycle. To verify this hypothesis, we generated MCF10A cells expressing a conditionally active form of HDAC7, fused to the ligand-binding domain of the estrogen receptor (Fig. 4A). This mutant, named HDAC7/SA-ER, presents the replacement of four serine residues with alanine residues, in the amino-terminal region (Dequiedt et al., 2003). In the wild-type protein, these serine residues, once phosphorylated by various kinases, become binding sites for 14-3-3 proteins and are required for the efficient nuclear export of the deacetylase (Yang and Seto, 2008). Hence, upon treatment with 4-OHT, this chimera should promptly accumulate in the nucleus and repress MEF2-dependent transcription. Fig. 4A shows that the fused protein is expressed at the expected size and, after 4-OHT addition, it efficiently accumulates in the nucleus of MCF10A cells (Fig. 4B). Moreover, HDAC7/SA-ER effectively repressed MEF2-dependent transcription, as evidenced by the decrease in RHOB mRNA levels after 8 days in 3D culture (Fig. 4C). To determine the effect of unscheduled HDAC7 repressive activity during morphogenesis, we observed the acinar size at 4, 8 and 12 days after induction with 4-OHT. Acini that had been generated by MCF10A cells expressing HDAC7/SA-ER were bigger at all time points analyzed (Fig. 4D). This increase in size depended on an increase in the number of nuclei per acinus (Fig. 4E). The increased number of acini presenting a partially filled lumen (Fig. 4F) and the quantitative analysis in Fig. 4G) confirmed the pro-growth effect of HDAC7. However, HDAC7/SA-ER acini were also characterized by higher numbers of picnotic and fragmented nuclei (Fig. 4E, arrows, and quantitative analysis in Fig. 4H) pointing to an increase in the apoptotic activity as a counterbalance in order to restrain excessive proliferation. These data indicate that although HDAC7 can stimulate epithelial cell growth, compensatory mechanisms are engaged that limit this potentially harmful effect. When the MEF2-binding sequence (amino acids 78–93) was removed, HDAC7/SA-ER was incapable of sustaining cell proliferation (Fig. S2A–E). These results further strengthen the evidence for the importance of the MEF2–HDAC axis in the control of mammary epithelial cell proliferation.

**MEF2-dependent transcription and HDAC7 levels are regulated during growth arrest in mammary epithelial cells**

The discovery that MEF2 activity influences cell cycle progression encouraged us to address whether this response can also be observed independently from the morphogenetic process. As alternative conditions for G0 induction, exit from the cell cycle was promoted by density-dependent inhibition and by growth factor starvation. Under contact inhibition, MEF2A and MEF2D protein levels increased in the absence of appreciable rises of the relative mRNAs (Fig. 5A and B). As described above under 3D culture conditions, HDAC7 protein levels decreased and HDAC5 downregulation (Fig. S1A).

** Unscheduled expression of MEF2 compromises cell proliferation and reduces acini size**

To understand the contribution of the MEF2–HDAC axis, we decided to use an alternative strategy. We introduced into MCF10A cells a conditionally active form of MEF2C fused to the VP16 activation domain of herpes simplex and to the ligand binding domain of the estrogen receptor (Flavell et al., 2006), as a control, a DNA-binding defective mutant (DBD) was used (Fig. 3A). In 2D culture, activation of MEF2–VP16 with 4-hydroxysteramoxifen (4-OHT), but not the control construct, efficiently promoted transcription of MEF2-target genes (Fig. 3B). Importantly, the magnitude of induction perfectly mirrored the changes observed during acini morphogenesis in 3D culture (compare Figs 2B and 3B). Induction of MEF2 transcriptional program in 2D-cultured MCF10A cells had a profound effect on proliferation (Fig. 3C). This anti-proliferative effect was also observed when cells were grown in 3D. Using a timecourse analysis we indicated that stimulation of MEF2 activity within the first four days constrained acinar size (Fig. 3D). At day 4, in the presence of unscheduled MEF2 transcriptional activity, acinar structures were smaller and populated by a reduced number of cells (Fig. 3E and F). To understand whether MEF2 elicits a growth-repressive effect during different phases of acinar morphogenesis, we selectively stimulated its activity for 4 days, starting from day 4, 8 or 12. Boosting MEF2 transcriptional activity from day 4 to day 8 still interfered with the proliferation of MCF10A cells, as evidenced by the reduced acini size (Fig. 3G), the number of cells (Fig. 3H) and the dramatic decrease of mitotic activity (Fig. 3I). It is noteworthy that activation of MEF2 at the next time intervals (at day 8 or 12) did not exert any effect on acinar diameter (Fig. 3J and K), cell numbers or luminal mechanisms, as observed in other contexts (Liu et al., 2014). In fact, MCF10A cells with downregulated MEF2D exhibited an increase in MEF2A protein levels, in concert with HDAC4 and HDAC5 downregulation (Fig. S1A).
levels increased (Fig. 5A). Expression of KLF2, RHOB and HDAC5 augmented, whereas HDAC7 mRNA levels were unchanged (Fig. 5B). Similar results were obtained when G0 was elicited through serum and growth factor deprivation (Fig. 5C and D). Serum starvation triggered the downregulation of HDAC4, as previously observed (Cernotta et al., 2011). Induction of HDAC7 repressive activity using the HDAC7/SA-ER chimera in cells that had been subjected to growth factor deprivation limited the
Fig. 3. See next page for legend.
upregulation of RHOB expression (Fig. S3A), sustained cell proliferation (Fig. S3B) and DNA synthesis (Fig. S3C). An increase of cells in S-phase could also be achieved using a conditionally active mutant form of HDAC4 (Fig. S3B). Compared to EGF, the HDAC7/SA-ER chimera was less capable of repressing RHOB and sustaining DNA synthesis. This partial effect could be a result of the dramatic decrease in its expression that is observed when cells were deprived of serum and growth factors (Fig. S3D,E).

The most prominent effect of cell cycle exit on the MEF2–HDAC axis, in both 3D and 2D culture models, was the downregulation of HDAC7 levels. To verify whether the upregulation of MEF2-target genes depends on the reduced recruitment of HDAC7 on the promoter of such genes, we performed chromatin immunoprecipitation (ChIP) analysis. The RHOB promoter was selected as a prototype of the MEF2-target genes. Fig. 5E illustrates that HDAC7 bound to the RHOB proximal promoter during the proliferative phase, whereas this binding was clearly reduced upon growth arrest, as induced by either serum starvation or density-dependent inhibition.

HDAC7 binds to the p21 promoter

Cyclin-dependent kinases (CDKs) are master regulators of cell cycle progression and cell proliferation. The CDK inhibitor p21 (CDKN1A) is a well-known negative regulator of proliferation, under the control of multiple signals, including growth factors and differentiation (Besson et al., 2008). Some reports have suggested that class-IIa HDACs are involved in the regulation of transcription of CDKN1A (Liu et al., 2009; Mottet et al., 2009; Wilson et al., 2008). To explore whether MEF2s and class-IIa HDACs control p21 expression during acinar morphogenesis, we initially interrogated the ENCODE database to map the epigenetic status of the CDKN1A promoter in human mammary epithelial cells (HMECs) (Ernst et al., 2011; Rosenbloom et al., 2013). We investigated the genomic region that is situated between two insulators (CTCF). Trimethylated Lys4 of histone 3 (H3K4me3) and acetylated Lys27 of histone 3 (H3K27ac), well-known markers of open chromatin status and characteristics of active promoter and enhancer regions, outline the first intron as an important element that is involved in CDKN1A transcription (Fig. 6A). A similar pattern can be observed in lymphoblastoid GM12878 cells (Fig. S4) and in the leukemia cells K562 (data not shown). Interestingly, the available ENCODE data for GM12878 and K562 cell lines highlighted the conserved binding of MEF2 factors to the promoter and enhancer elements, although with slight variations in the position of binding.

Next we screened for potential MEF2-binding sites a 20-kb genomic region of CDKN1A, comprising the first intron, which contains the active chromatin markers above described. The enlargement in Fig. 6A illustrates the presence of several potential MEF2 binding sites in the analyzed genomic region and particularly in the first intron.

To evaluate the involvement of HDAC7 in the regulation of CDKN1A transcription, we decided to investigate its ability to bind to the regions highlighted in Fig. 6A. ChIP experiments proved that HDAC7 bound preferentially to the region containing the MEF2-binding site at +1.5 kb from the transcription start site (TSS). Importantly, this binding was abolished or dramatically reduced when growth arrest was induced by growth factor starvation or high confluence (Fig. 6B).

Class-IIa HDACs are recruited to specific genomic regions following interactions with DNA-binding transcription factors. Hence, we assessed the binding of MEF2D to the same genomic regions. In proliferating cells, MEF2D bound to the RHOB and the CDKN1A promoters at +1.5 and +2.1 kb from the TSS, respectively (Fig. 7A). Surprisingly, a different behavior was observed in stably transfected cells as compared to confluent cells. MEF2D binding was maintained under starvation on both promoters in the same regions (slightly increased in the case of CDKN1A), whereas it was dramatically decreased in confluent cells. For CDKN1A, a new binding of MEF2D at the −5.7 kb region was observed only when cells were arrested through confluence.

ChIP experiments using an antibody against H3K27ac (Fig. 7B) confirmed the ENCODE data. Enrichment for H3K27 acetylation could be observed from +1.5 kb up to +3.6 kb from the TSS of the CDKN1A promoter (compare Fig. 6A). Concerning RHOB, H3K27ac was not pronounced, probably because this region is very close to the TSS, as reported in the ENCODE project. Under starvation, a consistent increase in H3K27ac was observed for the RHOB promoter, and this increase was much less pronounced at confluence. Overall, changes in this epigenetic modification are in agreement with MEF2D and HDAC7 binding to promoters.

Growth arrest through starvation elicits an overt increase of H3K27ac around the +2.1-kb region of the CDKN1A promoter. A small but significant increase can be appreciated also in the region +1.5 kb, in accordance with MEF2D binding and HDAC7 release. When growth arrest was induced through high confluence, the most
evident relative increase in H3K27ac regarded the overall poorly acetylated region at −5.7 kb. This is the same region bound by MEF2D in confluent cells (Fig. 7A).

The involvement of the MEF2–HDAC axis in the regulation of CDKN1A transcription was confirmed after induction of the MEF2-VP16 chimera in 2D conditions at both mRNA and protein levels.
**Fig. 5. MEF2-dependent transcription and HDAC7 levels are regulated during growth arrest in mammary epithelial cells.** (A) Immunoblot analysis of MEFs and class-IIa HDAC-family member levels in MCF10A cells. Cellular lysates were obtained at the indicated time points after seeding in complete medium and subjected to immunoblot analysis using the specific antibodies. AKT1 was used as loading control. Cell cycle analysis is provided underneath the blots as percentage of cells. (B) qRT-PCR analysis was performed to quantify mRNA levels of class-IIa members (HDAC4, HDAC5 and HDAC7) and of MEF2s (MEF2A and MEF2D), as well as of MEF2-target genes (KLF2 and RHOB) in MCF10A cells. The fold induction was calculated as the ratio relative to expression at 1 day of culture in complete medium. Data are from three independent experiments. (C) Immunoblot analysis of MEF2- and class-IIa-HDAC-family member levels in MCF10A cells. Cellular lysates were obtained at the different growing conditions and subjected to immunoblot analysis using the specific antibodies. AKT1 was used as loading control. Cell cycle analysis is provided underneath the blots as percentage of cells. (D) qRT-PCR analysis was performed to quantify the mRNA levels of the most expressed class-IIa members (HDAC4, HDAC5 and HDAC7) as well as of MEF2 members (MEF2A and MEF2D) and MEF2-target genes (KLF2 and RHOB) in MCF10A cells. Fold induction was calculated as the ratio between starvation and growing conditions. Data are from three independent experiments. (E) qRT-PCR analysis on chromatin that had been immunoprecipitated with the indicated antibodies in the different conditions shown. Mean±s.d.; *P<0.05, **P<0.01, ***P<0.005 (one-way ANOVA test coupled to Tukey’s HSD post-hoc test).

**DISCUSSION**

The mammary gland undergoes complex remodeling during different stages – i.e. puberty, pregnancy, lactation and involution (Gjorevski and Nelson, 2011). 3D cultures have significantly helped in understanding how some of these processes take place.
Particularly, although MCF10A cells grown under 3D conditions do not enter terminal differentiation (Muthuswamy et al., 2001) with milk protein production, the imposed 3D environment influences cell behavior and mimics the steps required for proper developmental and homeostatic cues (Leung and Brugge, 2012; Lo et al., 2012; Shaw et al., 2004). Complex cellular decisions take place during the 16-day interval that is necessary for acini formation. After the initial proliferation phase, cells begin to polarize and gradually exit from the cell cycle. The process terminates with the death of the cells in the lumen and with cavity formation (Frisch et al., 2013).

In this context, we have studied the regulation of the MEF2–HDAC axis and have dissected its contribution to the epithelial morphogenetic program. Quantitative and qualitative changes in gene expression profiles characterize the morphogenetic process of MCF10A cells grown under 3D condition (Yu et al., 2012). However, few studies have addressed the contribution of specific transcription factors and of the relative transcriptional gene networks to this morphogenetic process. We have initially demonstrated that the expression of MEF2-target genes is augmented during acini formation. This behavior resembles that under other differentiation contexts, as in skeletal muscle, where the contribution of the axis has been previously studied (McKinsey et al., 2000; Sebastian et al., 2013). Activation of MEF2 transcription is parallel to a dramatic downregulation of HDAC7 protein. In MCF10A cells, in contrast to other cellular models, antiproliferative signals unleash MEF2-dependent transcription by impacting on HDAC7 levels, rather than by controlling its nuclear–cytoplasmic shuttling (McKinsey et al., 2000; Paroni et al., 2008). HDAC7 downregulation is linked to growth arrest, and it can also be observed in 2D conditions, after growth factor starvation or density inhibition. At the moment, the mechanism that is responsible for HDAC7 downregulation is unknown. We can only exclude changes in mRNA levels, in proteasomal degradation and in autophagy.

HDAC5 and HDAC9 expression increases during acini formation. Being both MEF2-target genes, it is possible that such induction is part of the well-known negative feedback loop that is activated by MEF2 itself (Haberland et al., 2007). Nevertheless, because HDAC7 is the most expressed class-IIa deacetylase in breast tissue and in MCF10A cells, its withdrawal can justify the upregulation of MEF2 transcription. Furthermore, some reports indicate a differential repressive capability of class-IIa HDACs towards MEF2 proteins, with HDAC5 being the enzyme that is least prone to such repression (Dressel et al., 2001).
We exploited different approaches to interfere with the MEF2 transcriptional activity. In all instances, the effect was an impediment of the proliferative phase. Increasing MEF2-dependent transcription reduced cell division, whereas increasing HDAC7 repressive activity favored cell proliferation. The polarization process and the induction of cell death were unaffected after modulation of the MEF2–HDAC axis.

Although MEF2 proteins are the best-characterized class-IIa HDAC partners, these deacetylases can influence the activity of additional transcriptional regulators (Clocchiatti et al., 2013a). In this study, we have found logical correlations between MEF2 and class-IIa HDACs in the regulation of proliferation and gene expression during acini formation. Furthermore, deletion of the MEF2-binding sequence abrogated the pro-growth effect of...
Fig. 8. See next page for legend.
the nuclear resident HDAC7. Overall, our data strongly support that the described HDAC7 action is explicited through MEF2s.

We propose that the influence of the axis on cell proliferation could be operated through the regulation of the CDK inhibitor p21/Cdkn1A. Previous results have shown that class-Ia HDACs can control Cdkn1A expression (Liu et al., 2009; Mottet et al., 2009; Saramaki et al., 2009; Wilson et al., 2008); however, the mechanisms involved are debated. MEF2-binding sites are present in the genomic regions surrounding Cdkn1A. In particular, several MEF2-binding sites lie within the first intron. This intron is characterized by the presence of open chromatin markers (Ernst et al., 2011). Our results suggest that the MEF2–HDAC axis imposes a control on the cell cycle by modulating Cdkn1A expression. Recruitment of HDAC7 onto the Cdkn1A promoter is confined in a region (+1.5 kb) that is also under the control of MEF2 factors in other cell lineages scrutinized by the ENCODE project (Ernst et al., 2011). We have also proved that, in MCF10A cells, MEF2D binds to this region. However, MEF2D can also tether to additional regions, such as that at +2.1 kb, which are not bound by HDAC7, as we have recently observed in fibroblasts (Di Giorgio et al., 2015b). Whether specific MEF2–HDAC complexes exist bound to different chromatin regions deserves further studies.

At the +1.5 kb position, HDAC7 binding is dramatically reduced under conditions that stimulate cell cycle arrest, and this reduction is associated only with a modest increase in H3K27ac. Even though class-Ia HDACs do not exhibit catalytic activity, they can act as platforms to recruit class-I enzymes (Lahn et al., 2007; Di Giorgio et al., 2015a). Hence, HDAC7 could influence epigenetic changes at the Cdkn1A promoter both by recruiting co-repressors and by competing with co-activators for binding to MEF2 proteins. Certainly, it is possible that different growth conditions influence additional epigenetic changes within this genomic region.

The most evident increase in H3K27ac was observed as occurring at the +2.1 kb position when growth arrest was induced by starvation, pointing to the existence of additional mechanisms of regulation. Finally, our analysis of the binding of MEF2D to the Cdkn1A promoter indicates the existence of specific chromatin changes in response to different conditions of growth arrest.

The effect of MEF2 on the regulation of Cdkn1A transcription seems to be a general phenomenon that is not limited to MCF10A cells. Also, in fibroblasts, MEF2C and MEF2D bind to the Cdkn1A promoter. Importantly, in these cells, the downregulation of MEF2D is sufficient to increase cell proliferation and efficiently impacts on the expression of MEF2-target genes, including Cdkn1A (Di Giorgio et al., 2015b).

HER2 overexpression, a frequent oncogenic event in breast tumors, is able to maintain HDAC7 levels and decrease MEF2 transcription during the morphogenetic process, which might be an additional and powerful way to control p21 function beyond regulating its localization (Xia et al., 2004). Interestingly, forcing MEF2 activity in MCF10A HER2-overexpressing cells reverses the transformed phenotype, mainly by restraining proliferation, thus ensuring correct epithelial organization. Furthermore, treatment with the receptor tyrosine kinase (RTK) inhibitor lapatinib rescued HDAC7 downregulation and acini formation in 3D cultured cells. In conclusion, our studies unveil a role for the MEF2–HDAC axis in the control of epithelial cell proliferation and suggest that targeting class-Ia HDACs with inhibitors could be an interesting therapeutic strategy, in addition to or in combination with treatment with RTK inhibitors, for the treatment of breast cancer.

MATERIALS AND METHODS

Cell culture and reagents
Human normal immortalized epithelial breast cells (MCF10A) (Whyte et al., 2010) and MCF10A cells expressing HER2 were maintained in Ham’s F12: Dulbecco’s modified Eagle’s medium (DMEM) 1:1 medium (Sigma-Aldrich) supplemented with 5% horse serum (Gibco), penicillin (100 U/ml), streptomyocin (100 µg/ml), t-glutamine (2 mM) (Lonza), insulin (0.01 mg/ml), hydrocortisone (500 ng/ml), cholera toxin (100 ng/ml) (Sigma-Aldrich) and epithelial growth factor (20 ng/ml) (Peprotech). HEK-293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), t-glutamine (2 mM), penicillin (100 U/ml), and streptomyocin (100 µg/ml) (Lonza). Cells expressing inducible forms of MEF2 and HDAC7 were grown in complete F12/DMEM medium without phenol red (Sigma-Aldrich) and with 5% charcoal-stripped horse serum. The proteasome inhibitor bortezomib (LC Laboratories) was used at 1 µM for 8 h. The lysosome and autophagy inhibitor chloroquine (Sigma-Aldrich) and with 5% charcoal-stripped horse serum. The proteasome inhibitor bortezomib (LC Laboratories) was used at 1 µM for 8 h. The lysosome and autophagy inhibitor chloroquine (Sigma-Aldrich) and with 5% charcoal-stripped horse serum. The proteasome inhibitor bortezomib (LC Laboratories) was used at 1 µM for 8 h. The lysosome and autophagy inhibitor chloroquine (Sigma-Aldrich) and with 5% charcoal-stripped horse serum. The proteasome inhibitor bortezomib (LC Laboratories) was used at 1 µM for 8 h. The lysosome and autophagy inhibitor chloroquine (Sigma-Aldrich) and with 5% charcoal-stripped horse serum. The proteasome inhibitor bortezomib (LC Laboratories) was used at 1 µM for 8 h. The lysosome and autophagy inhibitor chloroquine (Sigma-Aldrich) and with 5% charcoal-stripped horse serum.
Plasmid construction, transfection, retroviral and lentiviral infection, and silencing

The pWZL-Hygro-MEF2C-VP16-ER construct comprising the first 119 amino acids of MEF2C has been previously described (Di Giorgio et al., 2013). The ligand-binding domain of the estrogen receptor was PCR amplified from pcDNA MEF2-VP16-ER (Flavell et al., 2006) and cloned into pWZL-Hygro. To generate pWZL-Hygro-HDAC7/SA-ER, an EcoRI-digested fragment of the HDAC7/SA point mutant (Di Giorgio et al., 2013) was cloned into pWZL-Hygro-ER. MCF10A cells expressing the MEF2-VP16-ER or HDAC7/SA-ER transgenes were generated by using retroviral infection, as described previously (Cermotta et al., 2011). The HDAC7/SA-ER construct that comprised deletion of the MEF2-binding domain, lacking amino acids 78–93 was generated by using PCR and subsequent two steps cloning into pWZL-Hygro-ER. HEK-293T packaging cells were transfected 24 h after plating by using calcium-phosphate precipitation. After 48–72 h, the virus-containing medium was filtered and added to target cells. Recombinant lentiviruses (Sigma-Aldrich) were produced through transfection of HEK-293T cells. Briefly, subconfluent HEK-293T packaging cells were co-transfected with 20 µg of lentiviral vector plasmids, 15 µg of pCMV-A-R8.91 and 5 µg of VSVG envelope plasmid by using calcium-phosphate precipitation. After 24 h, medium was changed, and recombinant lentiviruses vectors were harvested 24–36 h later.

Immunofluorescence, antibody production and immunoblotting

Cells were fixed in 3% paraformaldehyde and permeabilized with 1% Triton-X100. Next, cover-slips were incubated with the following primary antibodies: anti-HDAC4 (Paroni et al., 2004), anti-HDAC7 (sc-74563; Santa Cruz Biotechnology), anti-MEF2D (610774; BD Transduction Laboratories) and anti-GM130 (610822; BD Transduction Laboratories). Then, they were incubated with 488- or 546-Alexa-Fluor-conjugated secondary antibodies (Life Technologies). Finally, cover-slips were incubated for 15 min with 5 µM TOPRO-3 (610822; Life Technologies) to label nuclei. Cells were imaged with a Leica confocal scanner SP. 

Mouse anti-BrdU (B8434; Sigma-Aldrich) was used as primary antibody. Beads and inputs were treated with proteinase K overnight at 68°C for degradation and reverse cross-linking. Genomic DNA was finally purified with Qiagen QIAquick PCR purification kit and eluted in 100 µl of water.

The graphic representation of the CDKN1A locus and its chromatin organization in human mammary epithelial cells was obtained from UCSC Genome browser (http://genome.ucsc.edu/). Analysis of the putative binding site for MEF2D was performed using JASPAR (jarpan.genereg.net).

Gene set enrichment analysis

Analyses were performed using the GSEA software (http://www.broadinstitute.org/gsea/index.jsp). The list of MEF2 target genes was obtained from the Molecular Signature Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). The dataset for MCF10A cells was obtained from GEO (http://www.ncbi.nlm.nih.gov/geo/) GSE26148 (Simpson et al., 2011). Human normal and breast tumor samples were taken from Curtis et al. (2012) with accession number EGA500000000083, deposited at the European Genome-Phenome Archive (EGA; http://www.ebi.ac.uk/ega/).

Statistics

Results were expressed as the average±s.d. of at least three independent experiments, except for ChIP experiments where the s.e.m. was calculated. Results were expressed as the mean or median±s.d. of at least three independent experiments, except for ChIP experiments where the s.e.m. was calculated. Statistical analyses were performed using a Student’s t-test with the level of significance set at P<0.05. Data from 3D acinar area measurements were analyzed using the non-parametric Mann–Whitney test (Prism GraphPad Software). Data were from at least three independent experiments. *P<0.05; **P<0.01; ***P<0.005.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.C. co-designed and performed most of the experiments, aided in the design of the study and reviewed the paper prior to submission. C.B. co-designed most of the experiments was responsible for the conception and design of the study, wrote, edited and submitted the manuscript. E.D.G. performed most of the experiments for the rebuttal and reviewed the paper prior to re-submission. G.V. performed experiments and reviewed the paper prior to submission; G.D. provided reagents, aided in the 3D cell cultures, reviewed the paper prior to submission. A.S. and R.P. performed the bioinformatics analysis. V.C. performed experiments for the rebuttal.
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Supplementary information
Supplementary information available online at http://jcs.biologists.orglookup/suppl doi:10.1242/jcs.170357/DC1

References


Figure S1: Effects of MEF2D down-regulation on acinar morphogenesis.

A) Immunoblot analysis of MEF2s and class IIa HDACs family members levels, in MCF10A expressing two different shRNAs against MEF2D or a control shRNA. Cellular lysates were generated and probed with the indicated antibodies. Akt was used as loading control.

B) qRT-PCR was performed to evaluate mRNA levels of MEF2-target genes and of MEF2D in MCF10A cells expressing the shRNA15 or the control shRNA and cultured in 3D for 16 days.

C) Quantification of luminal filling per structure and acini polarization, scoring Golgi orientation, in MCF10A cells expressing the shRNA15 or the control shRNA and grown in 3D for 16 days.

D) Acinar area (µm²) of individual acini generated in 3D culture by MCF10A cells expressing the shRNA15 or the control shRNA at 4, 6 and 12 days, determined using Volocity 3D image analysis software, and plotted as a box plot. Data are from a representative set of three independent experiments in which ~ 250 acini per condition were measured.
Figure S2: MEF2 repression is required for the proliferative activity of HDAC7/SA-ΔMEF2-ER chimera.

A) Confocal pictures of MCF10A cells showing HDAC7/SA-ER and HDAC7/SAΔMEF2-ER nuclear accumulation after the induction with 4-OHT. HDAC7 was visualized using a specific antibody. Nuclei were stained with TOPRO-3. Images are shown in pseudocolours.

B) Immunoblot analysis of HDAC7 levels in MCF10A cells expressing HDAC7/SA-ER, HDAC7/SA-ΔMEF2-ER or the control gene. Expression of the chimeras was induced by treating cells with 4-OHT for 24h. Cellular lysates were generated and subjected to immunoblot analysis to verify the molecular size of the transgene using an anti-HDAC7 antibody. Actin was used as loading control.

C) Cellular lysates from MCF10A cells expressing the indicated transgenes and treated for 24 hours with 4-OHT were immunoprecipitated with an anti-HDAC7 antibody. Immunoblots were performed using anti-MEF2D (upper panel) or anti-HDAC7 antibodies (lower panel).

D) Acinar size of MCF10A cells, expressing the indicated transgenes, in 3D culture for 4, 8 and 12 days. When cells were seeded, 4-OHT was added to culture medium as well as every fourth day in culture. Data are from a representative set of three independent experiments in which ~250 acini per condition were measured.

E) Proliferation of cells expressing the different transgenes. MCF10A cells expressing the indicated transgenes and treated with 4-OHT were grown for the indicated times and counted. Data are presented as mean ± SD; n = 3.

F) mRNA expression levels of CDKN1A were measured by using qRT-PCR in MCF10A cells expressing the indicated transgenes grown in 2D culture. 4-OHT was added to the culture medium the day of seeding. Data are presented as mean ± SD; n = 3.
Figure S3. Class IIa HDACs influence cell proliferation in 2D cultures
A) mRNA expression levels of the MEF2-target gene RHOB were measured by using qRT-PCR in MCF10A cells expressing HDAC7/SA-ER or control, grown in 2D culture for 3 days. 4-OHT was added to culture medium the day of seeding. Deprivation of EGF was used to induce starvation.

B) Proliferation of cells expressing the different transgenes. MCF10A cells expressing the indicated transgenes and treated with 4-OHT were grown for the indicated times and counted. Data are presented as mean ± SD; n = 3.

C) 48h after seeding quantification of BrdU positivity of MCF10A cells grown in 2D under the indicated conditions and expressing cells indicated transgenes was performed. Data are presented as mean ± SD; n = 3.

D) Immunoblot analysis of HDAC7 levels in MCF-10A cells grown in 2D culture in the presence or absence of EGF (starved) expressing the HDAC7/SA-ER. HDAC7/SA-ER activity was induced by treating cells with 4-OHT for 24 h. Cellular lysates were generated and subjected to immunoblot analysis to verify the molecular size of the transgene using an anti-HDAC7 antibody. Actin was used as loading control.

E) qRT-PCR analysis of HDAC7/SA-ER mRNA levels in MCF10A cells grown in 2D culture in the presence or absence of EGF (starved) expressing the HDAC7/SA-ER.
Figure S4. Characterization of the epigenetic modifications and MEF2 binding in the CDKN1A genomic region.

A) Schematic representation of the epigenetic status of the CDKN1A locus in human lymphoblastoid GM12878 cells taken from ENCODE project. The analyzed region has been comprised by two insulator CTCF peaks and the promoter has been identified through

B)

C)
H4K4me3 and H3K27ac signals. Arrows mark MEF2 binding sites identified by ENCODE consortium.

B) Schematic representation of the MEF2A and MEF2C peaks in the CDKN1A locus in human lymphoblastoid GM12878 cells taken from ENCODE project. MEF2 factors associate with regions marking both promoter (H4K4me3) and enhancer (H3K4me1) elements.

C) HDAC7 down-regulation in HER2 cells following lapatinib treatment. Immunoblot analysis of HDAC7 levels in 2D culture of MCF10A cells expressing HER2. Cells were pre-treated for 12 hours with lapatinib (10µM) followed by 8 hours of incubation with bortezomib or bortezomib in association with chloroquine as indicated. p53 was used as positive control for proteasome-mediated degradation inhibition. p62 was used as positive control of blocking lysosomal/autophagy degradation. ERK as loading control and anti-phospho ERK for testing lapatinib activity.