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Epidrug Mediated Re-Expression of miRNA targeting the *HMGA* Transcripts in Pituitary Cells

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Short title: Re-Expression of HMGA target miRNA in pituitary

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Abstract

Transgenic mice overexpressing the high mobility group A (*HMGA*) genes, *Hmga1* or *Hmga2* develop pituitary tumours and their overexpression is also a frequent finding in human pituitary adenomas. In some cases, increased expression of *HMGA2* but not that of *HMGA1* is consequent to genetic perturbations. However, recent studies show that down-regulation of microRNA (miRNA), that contemporaneously target the *HMGA1* and *HMGA2* transcripts, are associated with their overexpression. In a cohort of primary pituitary adenoma we determine the impact of epigenetic modifications on the expression of HMGA-targeting miRNA. For these miRNAs, chromatin immunoprecipitations showed that transcript down-regulation is correlated with histone tail modifications associated with condensed silenced genes. The functional impact of epigenetic modification on miRNA expression was determined in the rodent pituitary cell line, GH3. In these cells, histone tail, miRNA-associated, modifications were similar to those apparent in human adenoma and likely account for their repression. Indeed, challenge of GH3 cells with the epidrugs, zebularine and TSA, led to enrichment of the histone modification, H3K9Ac, associated with active genes, and depletion of the modification, H3K27me3, associated with silent genes and re-expression of HMGA-targeting miRNA. Moreover, epidrugs challenges were also associated with a concomitant decrease in hmga1 transcript and protein levels and concurrent increase in bmp-4 expression. These findings show that the inverse relationship between HMGA expression and targeting miRNA is reversible through epidrug interventions. In addition to showing a mechanistic link between epigenetic modifications and miRNA expression these findings underscore their potential as therapeutic targets in this and other diseases.

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Introduction

Human pituitary adenomas are common intracranial neoplasms that show subtype-specificity, reflecting the phenotypic characteristics that define their cognate differentiated cellular counterparts. [1,2] However, their growth and endocrine characteristics are frequently exaggerated, and they fail to respond appropriately to homeostatic control and regulatory mechanisms that characterizes their normal cellular counterparts. [2]

In common with most other tumour types the genesis of pituitary tumours is consequent to the combined contribution of genetic and epigenetic aberration, targeting the genome and epigenome respectively.[3,4,2,5] The relative contributions of these aberrations, their order of appearance, and the way in which their effects are manifest are largely unknown, however, specific aberration, be they genetic or epigenetic, are either common or particular to an adenoma subtype(s). [6,5,7] Moreover, the demarcation between genetic and epigenetic aberrations are perhaps less precise than initial findings suggested. In this context, we now know that the *GNAS1* gene (the *gsp* oncogene), is activated or inappropriately expressed, either through mutation or relaxation of imprinting in growth hormone secreting pituitary adenomas. [8] Similarly, early reports describing inappropriate expression of the high mobility group A gene (*HMGA2*) showed this to be associated with genetic change, apparent as chromosomal amplification or re-arrangement. [9,10] However, more recent investigations now describe association between microRNA (miRNA) and the inappropriate expression of *HMGA2* and also that of the *HMGA1* gene. [11] In these cases, increase in HMGA1 and HMGA2 expression is associated with loss or significantly reduced expression of miRNA that target these gene transcripts.

MiRNAs play important roles in a range of essential, albeit diverse, cellular processes, that include differentiation, cell growth and cell death. [12] They comprise a class of endogenous, non-protein coding, single-stranded RNAs, that range in size from 20 to 25 nucleotides and repress posttranscriptional protein synthesis by pairing to the 3′-untranslated regions of target mRNA(s) and, dependent on the integrity of the pairing, promotes degradation of the mRNA target transcript or

repress post-transcriptional translation. [13] Reports of their inappropriate expression in disparate tumour types is persuasive with respect to their role in tumourigenesis. In a pituitary tumour context several recent reports describe inappropriate miRNA expression patterns together with identification of their putative mRNA targets. The mechanistic potential of miRNAs to elicit tumour suppressor activity in a pituitary context was first reported by Palmieri and co-workers. [11] They identified loss or significantly reduced expression of multiple miRNAs that target the *HMGA1* and *HMGA2* transcripts across each of the major pituitary adenoma subtypes. Moreover, heterologous expression of these miRNA in pituitary cell lines inhibited cell proliferation, colony forming activity (CFE) and leads to decrease in cell viability. [11] In a follow on study, in this case interrogating GH secreting adenoma, multiple miRNA that target the HMGA and the E2F1 transcripts were also identified and their heterologous expression were also associated with similar end-points. [14] More recent studies, of inappropriate miRNA expression profile in this case in GH and ACTH secreting adenomas also show that particular miRNA can elicit growth suppressive actions *in vitro*, and also in, *in vivo* model systems. [15,16] Collectively, these reports portend the therapeutic potential of miRNA through either restoration or inhibition of their expression in tumour cells.

Despite a burgeoning literature describing the inappropriate expression of miRNAs in pituitary adenomas, and still more recent studies that reveal the impact of these non-coding RNAs on biologically relevant endpoints, our understanding of their transcriptional regulation *per se* is incomplete. However, as with conventional protein-coding genes, it is apparent that the same regulatory mechanisms regulate their expression. [17] In these cases, expression of miRNAs can be silenced through inappropriate hypermethylation of CpG islands and/or by histone modifications. [18] In this report we explore, for the first time, the potential impact of epigenetic modifications on the expression of miRNA that target the *HMGA1* and *HMGA2* transcripts in primary pituitary adenomas. Moreover, to determine the functional impact of epigenomic modifications on miRNA expression, epidrug challenges were employed to restore their expression and to explore their sequelae for the *HMGA1* transcript and its protein product.

We now show, in human pituitary adenomas that *HMGA*-targeting miRNA are principally repressed through histone modification that are commonly associated with gene silencing. Moreover, the rodent homologues of these miRNA are also repressed in association with similar histone tail modifications. Epidrug mediated reversal of these modifications restores their expression and is associated with depletion of their target transcript in these cells. The potential of pharmacological manipulation of the epigenome either alone or in combinations with more conventional therapeutic options offers exciting prospect for future medical management of these and other tumour types.

Materials and Methods

Cell Culture:

GH3 a rat pituitary cell line (CCL-82.1: American Type Culture Collection, Manassas, VA) in the somatolactotroph lineage were cultured in DMEM (Biosera, Ringmer, UK), supplemented with 10% fetal bovine serum, 4µg/mL Gentamycin (Sigma-Aldrich, Dorset, UK) and 2µg/mL Ampicillin (Sigma-Aldrich) in a 5% CO_2 atmosphere at 37°C and as previously described. [19] Cells in the current study had undergone less than 15 passages.

Primary Tissue:

For some of the described investigations we used, as controls, DNA and RNA extracted from normal rat pituitaries. The strain of these pituitaries was *Sprague Dawley*, and they were obtained from a commercial source (Charles River, Kent, UK).

Human Tissue Samples:

Primary pituitary adenomas, comprising each of the major subtypes were graded according to a modified Hardy classification as described previously. [20] The subtypes comprised, GH-secreting tumours (GH), all of which were grade 2 macroadenomas, Prolactinomas (PRL) were grade 1 microadenomas, and also grade 2 and 3 macroadenomas, Corticotrophinoma (ACTH) were grade 2 macroadenomas, Non-functioning adenomas (NF) were all grade 2 and 3 macroadenomas and their clinical characteristics have been described previously by us. [20] As control we used post-mortem normal pituitaries, acquired within 12 hours of death. However, a caveat associated with these *apparent normal* controls and indeed of the normal rat pituitary in the studies employing GH3 cells is that the comparisons represent essentially monoclonal (tumour cells) populations with an *admix* of cells that comprise the normal gland. Therefore, the findings and their interpretation should be viewed and regarded in the light of these limitations. Primary human tissue was stored at -80°C before use. Only those adenomas were tumour cells made up at least 80% of the specimen, as determined at surgery, and confirmed by neuropath logical assessment, were used in the study. Before the described

extraction protocols, tumours and normal pituitaries were freeze fractured using a biopulveriser (BioSpec Products, Inc. Bartlesville, OK) to achieve a homogenous mixture of cells. The absolute number of primary tumours analyzed for each of the variables is shown in the figures and comprised 5 GH, 5 PRL, 4 ACTH and 9 NF adenomas. Tumour tissue was obtained with informed consent, and all studies were performed with institutional ethical approval.

Chemicals:

The cytidine deaminase inhibitor and DNA demethylating agent, zebularine [1-(-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one], and histone deacetylase inhibitor, Trichostatin A (TSA) were obtained from Sigma-Aldrich. Stock solutions were prepared as previously described and stored in accordance to the manufacturer's instructions. [19] GH3 cells were seeded into six-well plates and incubated overnight in growth media and subsequently challenged for 48hrs with either zebularine, or TSA alone, or in combination at the doses shown in the figures and with a single refreshment after 24hrs. Cells were then harvested after a total of 72hrs in culture. All experiments were repeated thrice with triplicate determinations.

Bioinformatic identification of miRNA transcription start sites:

The miRNA selected for investigation in this study are those identified in a previous report, which also describes their specificity for the *HMGA1* and *HMGA2* transcripts in primary pituitary adenomas. [11] In these cases we focused our investigation on miR-16, miR-26b, mirR-34b miR-196a2 and let-7a (Table 1, supplemental data) since these miRNA have been shown to target *HMGA1* and *HMGA2* . In addition we included, as internal control, miR-320 that shows increased expression in GH secreting adenoma. [14] MiRNA sequences, their specific transcription start sites and, where present, their associated CpG islands, were determined using the, miRBase, miRStart and miRT databases. [21-23]

Expression Analysis:

MiRNA were extracted from, *Sprague Dawley* rat pituitaries, GH3 cells, primary pituitary adenomas and post-mortem normal pituitaries using the mirPremier miRNA extraction kit (Sigma-Aldrich) in accordance with the manufacturer's instructions and stored at -80°C prior to analysis.

Stem-Loop RT-PCR:

Reverse transcription for each of the mature miRNA [described above and first identified in [14,11]] was performed using stem-loop primers that were specific for each miRNA and also for RNU6B as endogenous control for quantitative RT-PCR expression and as described previously. [24,25] Stem-Loop primer sequence and their specific miRNA targets are described in supplemental Table 1 (Supplemental data). cDNA was synthesized using 200U of Maloney mouse leukemia virus reverse transcriptase (Promega, Southampton, UK) according to the manufacturer's instructions and as previously described. [26]

Quantitative RT-PCR:

The primer sequences for real time quantification are shown in Table 1 (Supplemental Table 1) using conditions previously described. [19] All samples were analyzed in triplicate to account for technical variation. The target gene transcripts for the miRNA were normalized to their respective endogenous controls (see above and Supplemental Table 1). For analysis of HMGA1 and BMP-4 expression, transcript expression was normalized to GAPDH. [24,27,11] Relative quantification was carried out using $2^{-\Delta\Delta CT\ threshold}$ method.

Bisulphite conversion and pyrosequencing:

DNA was extracted from normal rat pituitaries, the GH3 cell line, primary pituitary adenomas and post mortem normal pituitaries. For the cell lines experiments extractions were performed before and after drug manipulations as previously described. [28] Bisulphite conversions were performed using EZ DNA Methylation-Gold Kit and the converted DNA eluted from the column and stored at -20°C as previously described. [29] The methylation status of selected miRNA associated CpG islands were determined using primers within *bona fide* CpG islands associated with the individual miRNA and as

determined by miRStart and miRT analysis [22,23,21]. Sequences for analysis were imported into PyroMark Assay Design 2.0 software for primer design of sodium bisulphite-converted DNA (Qiagen, Crawley, UK) and in the majority of cases encompassed 5-8 CpG dinucleotides (Table 1, Supplemental data). PCR amplifications and pyrosequencing was performed as previously described by us. [6]

Chromatin Immunoprecipitation (ChIP) analysis for histone modifications:

ChIP analysis was performed employing ChIP-IT Express Enzymatic Kit (Active Motif, Rixensart, Belgium) following the manufacturer's instructions and as we described previously.[20] Briefly, after DNA-Protein crosslinking in 1% formaldehyde (Sigma Aldrich), cells or primary tissues were lysed in ice-cold buffer, supplemented with protease inhibitor cocktail and phenyl methane sulfonyl fluoride (PMSF), after which cells or primary tissues were homogenized to release the nuclei. After resuspension in digestion buffer, supplemented with inhibitor cocktail and an enzymatic shearing cocktail (Active Motif) the samples were centrifuged. The supernatant comprised the sheared chromatin for immune-precipitation and a fraction (10%) set aside as input chromatin prior to antibody affinity manipulations and subsequent reversal of crosslinking. The histone modification assessed on the basis of their antibody affinity were those specific for active gene expression, H3K9Ac [30] and for DNA methylation-independent gene silencing H3K27Me3 [31]. Antibodies were obtained from Abcam (Cambridge, UK) and have been described previously [19]. After overnight incubation, precipitation was achieved using protein-G-magnetic bead separation (Active Motif), as described previously. [19] After elution and reversal of cross linking the chromatin was subject to proteinase K digestion and DNA was purified using GenElute PCR (Sigma-Aldrich). DNA was probed for enrichment using primers specific for individual miRNA (supplemental Table 1) by quantitative PCR. In these cases primers were designed to regions associated with transcription start sites and identified employing the on line databases previously described. [23,22] Percentage enrichment were determined relative to input DNA and was calculated as $100 \times 2^{-(\text{CT adjusted input-CT immune-precipitated})}$. Input DNA CT was adjusted from 10% to 100% by subtracting 3.322 CT or Log_2.

HMGA1 Enzyme Linked Immunosorbant Assay (ELISA):

Quantitative expression of intracellular hmga1was determined by ELISA specific for the rodent homologue of HMGA1 (Cusabio Biotech Co., Wuhan, China) according to the manufacturer's protocol and following the epidrug challenges described above. Lysates, from each of the tissues (normal rat pituitaries) or cell pellets (GH3 cells), was first quantified using a Bicinchoninic assay and as described previously [32]. Briefly, 1.5 mg/mL total protein lysate was used for each determination and assessed against an internal standard curve of known hmga1 concentrations. Hmga1 standards and samples were pipetted into individual wells and incubated with a biotinylated anti-rat hmga1 antibody. After washing, to remove unbound biotinylated antibody, the bound antibody was detected by an horseradish peroxidase-conjugated streptavidin and 3,3′,5,5′ tetramethylbenzidine visualization strategy. After addition of the stop solution, the intensity of the color was measured at 450 nm. The assay has a reported sensitivity of approximately 7.8pg/mL, and all experiments were repeated at least three times.

RESULTS

Histone modifications associated with *HMGA* transcript-targeting miRNA in primary pituitary adenomas:

In a cohort of primary pituitary tumours, comprising each of the major adenoma subtypes we determined the expression status of miRNA shown previously to target the *HMGA1* and *HMGA2* gene transcripts in these tumour types. [11] For each of the adenomas we also determined the histone tail modification commonly associated with active, H3K9Ac, and repressed genes, H3K27Me3. The findings for, miR-26b, miR-196a-2 and let-7a are presented in Figure 1 and for miR-16 and miR34b as supplemental data (supplemental data, Figure, S1). In these cases and for each of the miRNA robust expression was apparent in the post- mortem normal pituitaries. The majority of adenomas, irrespective of their subtype, showed reduced expression for each of these miRNA (Figure 1A and supplemental Figure S1A) and similar findings for their expression have been reported previously.

[11] In those cases where we observed decrease in miRNA transcript levels, ChIP analysis showed, in the majority of cases, depletion of the modification associated with active genes, H3K9Ac, and enrichment for the modification associated with silences genes, H3K27me3, relative to post-mortem pituitaries (Figure 1B and 1C, and supplemental Figure S1B and S1C, respectively). Distinct from these findings, and in agreement with a previous report [14], increased expression of miR-320, confined to GH secreting adenoma was apparent (supplemental Fig. S1A). In this case, and in contrast to other adenoma subtypes, the histone modification were those commonly associated with active genes (supplemental Fig S1B and S1C).

Methylation status of CpG islands associated with *HMGA* transcript-targeting miRNA in primary pituitary adenomas:

We next investigated the methylation status of the promoter associated CpG islands for each of the miRNA (miR-16, miR-26b, miR34b, miR-196-a2 and Let-7a) by pyrosequence analysis and relative to post-mortem normal pituitaries. In these cases and where sufficient primary tumour was available for analysis, low levels of methylation, between 4-12%, were detected for each of these miRNA with the exception of miR-26b (Supplemental Table S2). In this case we found that, 7 of 15 adenomas (~46%) irrespective of subtype showed increase in methylation (16-60%) relative to post-mortem normal pituitaries (~8%). However, the exception to this finding was the GH adenomas where we did not detect increase in CpG island methylation (Supplemental Table S2).

Expression of HMGA transcript-targeting miRNA in GH3 cells:

To gain insight into the functional impact of epigenetic modifications on miRNA expression we used the rodent pituitary cell line, GH3. Since previous studies have shown expression of hmga1 but not that of hmga2 in these cells [11] we focused our studies on hmga1. Relative to normal pituitaries GH3 cells expressed significantly higher levels of *hmga1* transcript as determined by RT-qPCR analysis (Figure 2A), however, and as we have shown previously in this cell line, expression of the bmp-4 transcript was significantly reduced relative to normal pituitary counterparts. [20] Stem-loop

RT-qPCR showed significantly reduced expression of the rodent homologues of, miR-16, miR-26b, miR-34b miR-196a-2 and let-7a in GH3 cells relative to normal pituitaries (Figure 2B).

Epigenomic modifications associated with miRNA expression patterns in GH3 cells:

ChIP analysis of histone modifications, associated with active genes, H3K9Ac, and repressed genes, H3K27Me3, in GH3 cell, showed these to be similar to those we observed in human pituitary adenomas. The findings for, miR-26b, miR-196a-2 and let-7a are presented in Figure 3 and for miR-16 and miR-34b as supplemental data (supplemental data, Figure S2). For each of these miRNA reduced transcript expression is associated with depletion of the modification associated with active gene, H3K9Ac, and enrichment of the modification associated with silenced genes, H3K27Me3 (Figure 3B and 3C and supplemental Figure S2B and S2C respectively). For miR-320, the converse histone modifications are apparent and are consistent with the expression status of this miRNA in these cells (Supplemental Figure. S2B and-2C).

Methylation status of CpG islands associated with miRNA expression in GH3 cells:

For each of the miRNA investigated in this study (miR-16, miR-26b, miR34b, miR-196a-2 and Let-7a) and as described for primary adenomas we determined their methylation status. For the CpG islands associated miR-16, miR-26b and Let-7a there was no evidence for increased methylation (~5-8%) in GH3 cells relative to normal rat pituitaries (Supplemental Table S2). However, the miR-196a-2 and miR-34b associated CpG islands were methylated at ~80 and ~50% respectively (>80%) in GH3 cells. In these cases, drug challenges were ineffective or associated with marginal decrease in methylation at these miRNA associated CpG islands (Supplemental Table S2).

Epidrug induced expression of miRNA in GH3 cells:

GH3 cells were incubated, either alone or in combination, with an inhibitor of DNA methylation, zebularine and with the histone deacetylase inhibitor (HDACi), TSA. The findings for miR-26b, miR-196a-2 and let-7a are shown in Fig. 3A and for miR-16, miR-34b and miR-320 in supplemental Figure S2A. In general, single drug challenges were either ineffective or led to marginal increase in

miRNA expression; however, combined drug challenges effectively increase expression of each of the miRNA with the exception of miR-320. For each of the miRNA, where their expression was increased it was associated with enrichment of the histone tail modification associated with active genes, H3K9Ac (Figures 3B and supplemental Figure S2B), and depletion of the modification associated with silent genes, H3K27Me3 (Figures 3C and supplemental Figure S2C). Distinct from these findings, epidrugs did not increase expression of miR-320; indeed, we observed a marginal decrease in transcript expression. However, in this case, epidrug challenges did not lead to significant change in histone tail modification associated with either active genes or repressed genes. For those miRNA where we determined CpG island methylation we observed a marginal (~20%) decrease in methylation in cells challenged with combined drugs (data not shown). These studies support a functional relationship between epigenetic modification and silencing or significant reduced expression of miRNA in pituitary cells. The data also shows that expression of these miRNA can be restored through epidrug mediated reversal of histone modifications.

Epidrug induced, and miRNA mediated, suppression of HMGA1 expression in GH3 cells:

The effects of epidrug-induced expression of endogenous miRNAs on the *hmga1* transcript were assessed in GH3 cells. These studies (Figure 4A) show that drug challenges are associated with/responsible for a significant decrease in the *hmga1* transcript relative to vehicle challenged cells. Furthermore, and as we showed previously, these epidrug challenges induced expression of the *bmp-4* gene. In these experiments and as we showed previously epidrug challenges (at the doses employed) did not lead to decrease in cell viability. [20] In addition to determining effects of epidrug-induced expression of endogenous miRNAs on the *hmga1* transcript we also determined effect on hmga1 protein. For quantitative analysis we used an ELISA specific for rodent hmga1. Figure 4B shows, in wild type GH3 cells, increase in hmga1 in GH3 cells relative to normal rat pituitaries and these findings are consistent with the transcript data shown in panel A. However, in GH3 challenged with epidrugs we observed a marginal but statistically significant decrease in hmga1 protein relative to GH3 challenged with vehicle alone.

DISCUSSION

An expanding literature, germane to most tumour types, including those of pituitary origin, describes inappropriate expression of miRNA and apparent as either increase or decrease relative to their respective normal tissue counterparts. Although significant progress has been made toward identification of their target transcripts our understanding of the aberrations responsible for the inappropriate expression of miRNA *per se* are far from complete. However, and in common with protein-coding genes, miRNAs can be silenced through aberrant hypermethylation of CpG islands and/or by histone modifications. [18]

Irrespective of pituitary adenoma subtype a significant proportion overexpress *HMGA1* and *HMGA2* [33,10,34,35], indeed, in the cohort we now describe, similar conclusions are apparent (data not shown). Convincing evidence for the role of these genes, and their protein products, in tumour evolution are provided through studies in transgenic mice, where, enforced expression of either *hmga1* or *hmga2* leads to the development of pituitary adenomas. [36-38] Although inappropriate expression of *HMGA2* in a proportion of prolactinoma is associated with chromosome rearrangement or amplification [39] in other pituitary adenoma subtypes, overexpression of *HMGA2* and also that of *HMGA1,* are not associated with these types of genetic perturbations. However, in these cases, loss or significantly reduced expression of target-specific miRNA, which contemporaneously target each of these transcripts, has been described. [11] Given our previous observations, and in particular for the BMP-4 gene in this tumour types [20] it seemed intuitive to characterize the epigenome associated with the miRNA targeting the HMGA1 and HMGA2 gene transcripts. We now show, for the first time for each of these miRNA that loss or significantly reduced expression of, miR-16, miR-26b,

miR-34b miR196a-2 and let-7a is associated with histone tail modifications that are commonly associated with gene silencing. Contrary to these findings, expression of miR-320 is increased in GH secreting adenoma and depleted in other adenoma subtypes and similar findings of subtype specific expression patterns have been reported elsewhere. [14] Moreover, and reinforcing these observations we now show that increased expression of miR-320 is associated with histone modification that characterize transcribed gene. Collectively, these findings would suggest that histone tail modifications impact on chromatin remodeling and miRNA expression. However a caveat associated with these observations is that the majority of adenomas in our investigation were macroadenomas. Indeed, and in this context, a single report has described miRNA expression that correlates with adenoma size. [40] Although this represents a single report the association between miRNA expression profiles and adenoma size are perhaps worthy of more detailed investigation.

To directly assess the functional implication of histone tail modifications on miRNA expression we used the GH3 cell line as a model system. In this cell line and similar to our findings in primary adenomas, the rodent homologues of miR-16, miR-26b, miR-34b, miR196a-2 and Let-7a also show significantly reduced expression relative to normal pituitary and the contrary finding is apparent for miR-320. ChIP analysis GH3 cell again showed histone modifications similar to those apparent in primary human pituitary adenoma. Since we [19,41] and others [18,42-44] have used epidrugs to restore expression of endogenous, epigenetically silenced mRNA and that of miRNA respectively, we exploited this approach in this report. For the first time, in a pituitary context we found combined drug challenges induced expression of the miRNA. In these cases, expression is associated with a reversal of the characterized histone modification toward those associated with active gene. Conversely, and reinforcing these findings, epidrugs did not lead to significant change in miR-320 expression or in the histone modifications associated with either active or silenced genes. The association between, drug-induced re-expression of endogenously silenced miRNA and the enrichment of histone modification commonly associated with active genes strongly support an epigenetic mechanism as responsible for gene silencing.

As a functional end-pint we determined the effects of epidrug- induced re-expression of endogenous miRNAs on their target transcript in GH3 cells. However, in these cases and since these cells do not express, *hmga2* [11] we focused attention on *hmga1*. The studies showed that epidrugs, principally as combined challenges, are associated with a decrease in the *hmga1* transcript levels relative to cell treated with vehicle alone. As control in these experiments, and as we reported previously [20], epidrug challenges induced robust re-expression of the *bmp-4* gene. These findings would suggest that the decrease in *hmga1* is not a consequences of drug-mediated cytoxicity or a generalized consequences of epidrug-induced miRNA expression. While similar observations, with respect to decreased expression of *HMGA1* and *HMGA2* transcript, in MEG-01 cells and also for *hmga1* transcript in GH3 cell have been reported previously.[11] these authors employed heterologous expression vectors harbouring each of the precursor miRNA. [11] Moreover, in this and in subsequent reports ([14,45] they also provide evidence that enforced expression of miRNA impact on mRNA stability and inhibition of translation. Using a quantitative ELISA based assay, specific for rodent hmga1, our data also shows that epidrugs lead to decrease in hmga1 protein. Taken together these findings would support the conclusion that, epidrug-induced and miRNA-mediated decrease in hmga1 is consequent to hmga1 transcript degradation and inhibition of translation. However, although technical limitations precluded us from determining the relative or absolute contribution of each of these pathways, our conclusions are reinforced by the independent approaches that reach similar conclusions and described above. [11,14,45]

Thus far, investigations characterizing the inappropriate expression of miRNA in the genesis of pituitary adenomas has already led to the identification of subtype-specific miRNA profiles [46,47,16], identification of miRNA that show correlations with tumour size [40] or are predictive of tumor sensitivity and or response to therapeutic interventions. [47,16] and have been subject to recent and extensive review, [48-51]. Still more recent studies have focused on the target transcripts for these miRNA. Future studies and treatment strategies offer significant promise in the context of epidrug induced re-expression of silenced miRNA either alone or in combination with more conventional therapeutic options. However, whiles these types of approach, particularly in a pituitary context are

attractive their effectiveness, either alone or in combination with conventional therapies, has not thus far been assessed in patient or indeed in animal models. Thus, necessary studies, initially in animal models will be required in these cases to address not only efficacy but also specificity and potential issues of cytoxicity. Indeed, and in this context, some of these issues have been subject to recent review (52). More recent reports that in this case identify targets of miRNA that themselves play key roles or are central regulators of pathways that include, as example protein kinase Cδ and PTEN-AKT respectively might offer alternate indirect approaches [16,15]. In these cases investigators identified previously unknown, but what would appear to be novel targets that may represent pathways for more direct therapeutic interventions.

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Figure Legends

Figure 1. Expression and histone tail modifications associated with *HMGA*-targeting miRNA in human pituitary adenomas. (A) From left to right, quantitative RT-PCR of miR-26b, miR-196a-2 and let-7a in non-functioning (NF), prolactinoma (P), corticotrophinoma (A) and growth hormone secreting adenomas (GH). Expression is reported relative to the mean of three post-mortem pituitaries, where the mean value is expressed as equal to 1 and where the individual values were within 10% of each other. Each bar represents the mean value ± SEM from three independent experiments performed in triplicate. **(B)** Chromatin immunoprecipitation analysis (ChIP) for the histone tail modification associated with active genes, H3K9Ac. Adenomas, and three post-mortem normal pituitaries, are those shown in panel A and enrichment in each case is relative to input chromatin. **(C)** ChIP analysis of adenomas and post-mortem normal pituitaries shown in panels A and B, for the histone tail

modification associated with silenced gene, H3K27Me3. In panels B and C each bar represents the mean value ± SEM from three independent experiments performed in triplicate.

Figure 2. Expression of the hmga1 transcript, and of *hmga1*- and *hmga2*- targeting miRNA, in GH3 cells. (A) Quantitative RT-PCR analysis of *hmga1* and *bmp-4* transcript expression in GH3 cells. Expression is reported relative to the mean of three normal rat pituitaries (NRP), where the mean value is expressed as equal to 100%. Each bar represents the mean value ± SEM from three independent experiments performed in triplicate. **(B)** RT-qPCR analysis of, miR-16, miR-26b, miR-34b miR-196a-2 and let-7a in GH3 cells where each of the miRNA are shown relative to their expression in normal rodent pituitary counterparts, set at 100%,. Each bar represents the mean value ± SEM from three independent experiments performed in triplicate.

Figure 3 Epidrug mediated effects on *hmga1* targeting miRNA (A) From left to right, expression of miR-26b, miR-196a-2 and let-7a in GH3 cells as determined by RT-qPCR and relative to normal rat pituitaries (NRP). Expression was determined in the absence or presence of the epidrugs, zebularine (Zeb) and trichostatin A (TSA) either alone or in combination. Doses of drugs are shown on the x axis. Expression is reported relative to the mean of three normal rat pituitaries (NRP), where the mean value was expressed as equal to 100% and where each bar represents the mean value ± SEM from three independent experiments performed in triplicate. Panels **B** and **C** show the ChIP analysis of the cells shown in panel **A**, where panel **B** is the modification associated with active gene, H3K9Ac and panel **C** is the modification associated with silencing, H3K27me3. Each bar represents the mean value ± SEM from three independent experiments performed in triplicate. Data was analyzed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ *versus* vehicle challenged GH3 cells.

Figure 4 Epidrug mediated inhibition of *hmga1* expression in GH3 cells. (A) GH3 cells were challenged with epidrugs as described in Figure 3 and expression of the *hmga1* and *bmp-4* transcript determined by RT-qPCR analysis. **(B)** ELISA analysis for rodent hmga1 protein in NRP and GH3 cells following the challenges shown in the figure legend. Transcript expression is reported relative to

the mean of three normal rat pituitaries (NRP), where the mean value is expressed as equal to 100% and where each bar represents the mean value ± SEM from three independent experiments performed in triplicate. For the ELISA studies, statistical analysis is relative to the expression of hmga1 in GH3 vehicle control and where each bar represents the mean value ± SEM from three independent experiments performed in duplicate. In panels A and B, data was analyzed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ versus vehicle challenged GH3 cells. Normal rat pituitaries, unfilled bars. GH3 cells in the presence and absence of epidrugs, filled bars.

Supplemental Figures

Figure S1. Expression and histone tail modifications associated with *HMGA*-targeting miRNA in human pituitary adenomas. (A) From left to right, quantitative RT-PCR of miR-16, miR34b and miR-320 in non-functioning (NF), prolactinoma (P), corticotrophinoma (A) and growth hormone secreting adenomas (GH). Expression is reported relative to the mean of three post-mortem pituitaries, where the mean value was expressed as equal to 1. Each bar represents the mean value ± SEM from three independent experiments performed in triplicate. **(B)** Chromatin immunoprecipitation analysis (ChIP) for the histone tail modification associated with active genes, H3K9Ac. Adenomas, and three post-mortem normal pituitaries, are those shown in panel A and enrichment in each case is relative to input chromatin. **(C)** ChIP analysis of adenomas and post-mortem normal pituitaries shown in panels A and B, for the histone tail modification associated with silenced gene, H3K27Me3. In panels B and C each bar represents mean value ± SEM from three independent experiments performed in triplicate.

Figure S2 Epidrug mediated effects on *hmga1* targeting miRNA (A) From left to right, expression of miR-16, miR-34b and miR-320 in GH3 cells as determined by RT-qPCR and relative to normal rat pituitaries (NRP). Expression was determined in the absence or presence of the epidrugs, zebularine (Zeb) and trichostatin A (TSA) either alone or in combination. Doses of drugs are shown on the x axis. Expression is reported relative to the mean of three normal rat pituitaries (NRP), where the mean value was expressed as equal to 100% and where each bar represents the mean value ± SEM from

three independent experiments performed in triplicate. Panels **B** and **C** show the ChIP analysis of the cells shown in panel **A**, where panel **B** is the modification associated with active gene, H3K9Ac and panel **C** is the modification associated with silencing, H3K27me3. Each bar represents the mean value ± SEM from three independent experiments performed in triplicate. Data was analyzed for significance by one-way ANOVA with Dunnett's multiple comparison post-test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ *versus* NRP.

REFERENCES

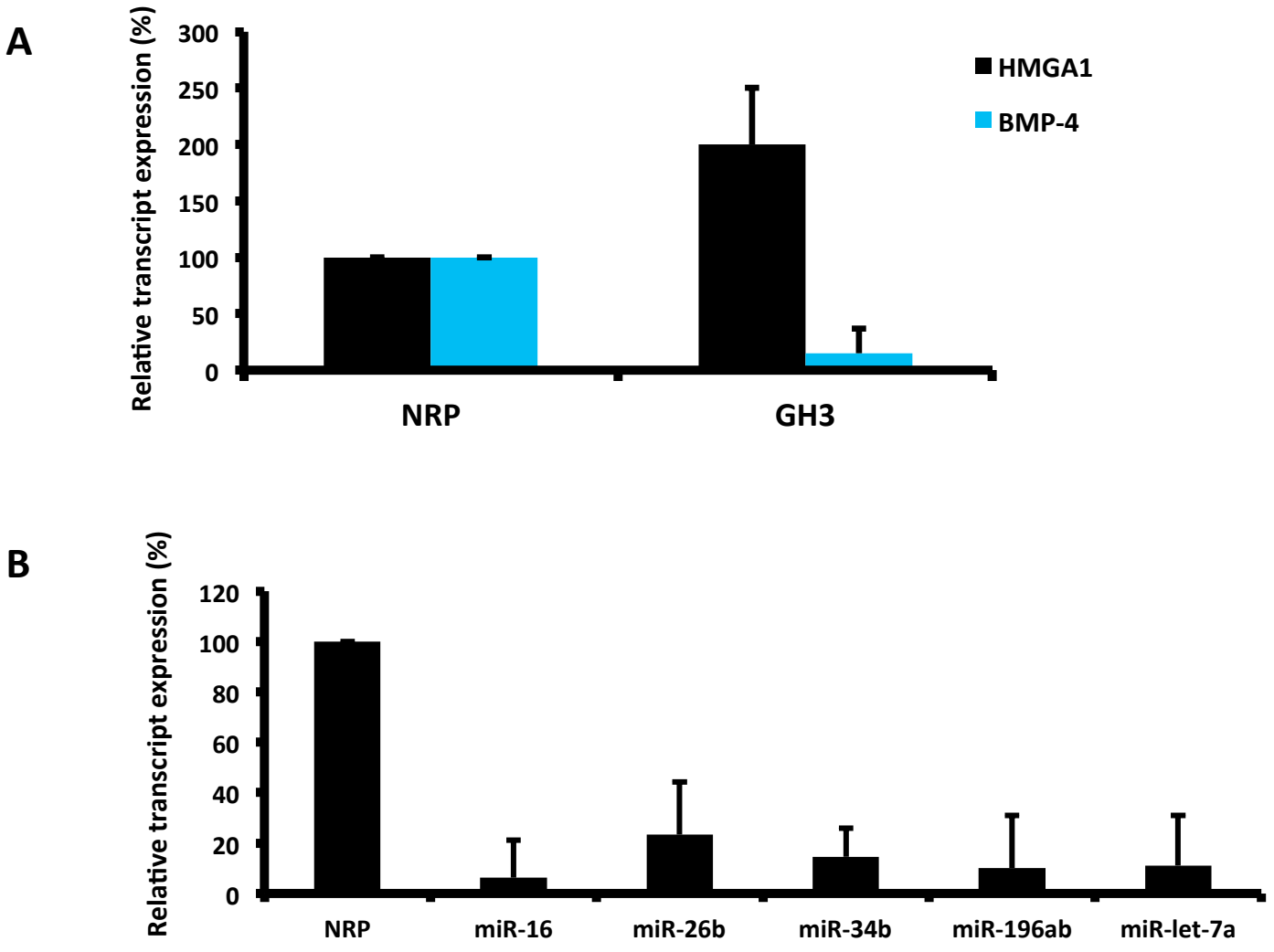
1. Melmed, S.: Mechanisms for pituitary tumorigenesis: the plastic pituitary. The Journal of clinical investigation **112**(11), 1603-1618 (2003). doi:10.1172/jci20401
2. Melmed, S.: Pathogenesis of pituitary tumors. Nature reviews.Endocrinology **7**(5), 257-266 (2011). doi:10.1038/nrendo.2011.40
3. Dudley, K.J., Revill, K., Clayton, R.N., Farrell, W.E.: Pituitary tumours: all silent on the epigenetics front. Journal of Molecular Endocrinology **42**(6), 461-468 (2009). doi:10.1677/jme-09-0009
4. Vandeva, S., Jaffrain-Rea, M.L., Daly, A.F., Tichomirowa, M., Zacharieva, S., Beckers, A.: The genetics of pituitary adenomas. Best practice & research.Clinical endocrinology & metabolism **24**(3), 461-476 (2010). doi:10.1016/j.beem.2010.03.001
5. Zhou, Y., Zhang, X., Klibanski, A.: Genetic and epigenetic mutations of tumor suppressive genes in sporadic pituitary adenoma. Mol Cell Endocrinol (2013). doi:10.1016/j.mce.2013.09.006
6. Duong, C.V., Emes, R.D., Wessely, F., Yacqub-Usman, K., Clayton, R.N., Farrell, W.E.: Quantitative, genome-wide analysis of the DNA methylome in sporadic pituitary adenomas. Endocr Relat Cancer **19**(6), 805-816 (2012). doi:10.1530/ERC-12-0251
7. Pease, M., Ling, C., Mack, W.J., Wang, K., Zada, G.: The role of epigenetic modification in tumorigenesis and progression of pituitary adenomas: a systematic review of the literature. PloS one **8**(12), e82619 (2013). doi:10.1371/journal.pone.0082619
8. Hayward, B.E., Barlier, A., Korbonits, M., Grossman, A.B., Jacquet, P., Enjalbert, A., Bonthron, D.T.: Imprinting of the G(s)alpha gene GNAS1 in the pathogenesis of acromegaly. The Journal of clinical investigation **107**(6), R31-36 (2001). doi:10.1172/jci11887
9. Fedele, M., Pierantoni, G.M., Visone, R., Fusco, A.: Critical role of the HMGA2 gene in pituitary adenomas. Cell cycle (Georgetown, Tex.) **5**(18), 2045-2048 (2006).
10. Finelli, P., Pierantoni, G.M., Giardino, D., Losa, M., Rodeschini, O., Fedele, M., Valtorta, E., Mortini, P., Croce, C.M., Larizza, L., Fusco, A.: The High Mobility Group A2 gene is amplified and overexpressed in human prolactinomas. Cancer Res **62**(8), 2398-2405 (2002).
11. Palmieri, D., D'Angelo, D., Valentino, T., De Martino, I., Ferraro, A., Wierinckx, A., Fedele, M., Trouillas, J., Fusco, A.: Downregulation of HMGA-targeting microRNAs has a critical role in human pituitary tumorigenesis. Oncogene **31**(34), 3857-3865 (2012). doi:10.1038/onc.2011.557

12. Zamore, P.D., Haley, B.: Ribo-gnome: the big world of small RNAs. Science **309**(5740), 1519-1524 (2005). doi:10.1126/science.1111444
13. Huntzinger, E., Izaurralde, E.: Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nature reviews. Genetics **12**(2), 99-110 (2011). doi:10.1038/nrg2936
14. D'Angelo, D., Palmieri, D., Mussnich, P., Roche, M., Wierinckx, A., Raverot, G., Fedele, M., Croce, C.M., Trouillas, J., Fusco, A.: Altered microRNA expression profile in human pituitary GH adenomas: down-regulation of miRNA targeting HMGA1, HMGA2, and E2F1. J Clin Endocrinol Metab **97**(7), E1128-1138 (2012). doi:10.1210/jc.2011-3482
15. Palumbo, T., Faucz, F.R., Azevedo, M., Xekouki, P., Iliopoulos, D., Stratakis, C.A.: Functional screen analysis reveals miR-26b and miR-128 as central regulators of pituitary somatomammotrophic tumor growth through activation of the PTEN-AKT pathway. Oncogene **32**(13), 1651-1659 (2013). doi:10.1038/onc.2012.190
16. Gentilin, E., Tagliati, F., Filieri, C., Mole, D., Minoia, M., Rosaria Ambrosio, M., Degli Uberti, E.C., Zatelli, M.C.: miR-26a plays an important role in cell cycle regulation in ACTH-secreting pituitary adenomas by modulating protein kinase Cdelta. Endocrinology **154**(5), 1690-1700 (2013). doi:10.1210/en.2012-2070
17. Fabbri, M., Calin, G.A.: Epigenetics and miRNAs in human cancer. Adv Genet **70**, 87-99 (2010). doi:10.1016/B978-0-12-380866-0.60004-6
18. Saito, Y., Liang, G., Egger, G., Friedman, J.M., Chuang, J.C., Coetzee, G.A., Jones, P.A.: Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer cell **9**(6), 435-443 (2006). doi:10.1016/j.ccr.2006.04.020
19. Al-Azzawi, H., Yacqub-Usman, K., Richardson, A., Hofland, L.J., Clayton, R.N., Farrell, W.E.: Reversal of endogenous dopamine receptor silencing in pituitary cells augments receptor-mediated apoptosis. Endocrinology **152**(2), 364-373 (2011). doi:10.1210/en.2010-0886
20. Yacqub-Usman, K., Duong, C.V., Clayton, R.N., Farrell, W.E.: Epigenomic Silencing of the BMP-4 Gene in Pituitary Adenomas: A Potential Target for Epidrug-Induced Re-expression. Endocrinology(Journal Article) (2012). doi:10.1210/en.2012-1231
21. Griffiths-Jones, S., Saini, H.K., van Dongen, S., Enright, A.J.: miRBase: tools for microRNA genomics. Nucleic Acids Res **36**(Database issue), D154-158 (2008). doi:10.1093/nar/gkm952
22. Bhattacharyya, M., Das, M., Bandyopadhyay, S.: miRT: a database of validated transcription start sites of human microRNAs. Genomics, proteomics & bioinformatics **10**(5), 310-316 (2012). doi:10.1016/j.gpb.2012.08.005
23. Chien, C.H., Sun, Y.M., Chang, W.C., Chiang-Hsieh, P.Y., Lee, T.Y., Tsai, W.C., Horng, J.T., Tsou, A.P., Huang, H.D.: Identifying transcriptional start sites of human microRNAs based on high-throughput sequencing data. Nucleic Acids Res **39**(21), 9345-9356 (2011). doi:10.1093/nar/gkr604
24. Kramer, M.F.: Stem-loop RT-qPCR for miRNAs. Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.] **Chapter 15**, Unit 15 10 (2011). doi:10.1002/0471142727.mb1510s95
25. Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Nguyen, J.T., Barbisin, M., Xu, N.L., Mahuvakar, V.R., Andersen, M.R., Lao, K.Q., Livak, K.J., Guegler, K.J.: Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res **33**(20), e179 (2005). doi:10.1093/nar/gni178
26. Bahar, A., Simpson, D.J., Cutty, S.J., Bicknell, J.E., Hoban, P.R., Holley, S., Mourtada-Maarabouni, M., Williams, G.T., Clayton, R.N., Farrell, W.E.: Isolation and characterization of a novel pituitary tumor apoptosis gene. Molecular endocrinology (Baltimore, Md.) **18**(7), 1827-1839 (2004). doi:10.1210/me.2004-0087

27. Peltier, H.J., Latham, G.J.: Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. Rna **14**(5), 844-852 (2008). doi:10.1261/rna.939908
28. Revill, K., Dudley, K.J., Clayton, R.N., McNicol, A.M., Farrell, W.E.: Loss of neuronatin expression is associated with promoter hypermethylation in pituitary adenoma. Endocr Relat Cancer **16**(2), 537-548 (2009). doi:10.1677/ERC-09-0008
29. Dudley, K.J., Revill, K., Whitby, P., Clayton, R.N., Farrell, W.E.: Genome-wide analysis in a murine Dnmt1 knockdown model identifies epigenetically silenced genes in primary human pituitary tumors. Molecular cancer research : MCR **6**(10), 1567-1574 (2008). doi:10.1158/1541-7786.mcr-08-0234
30. Jenuwein, T., Allis, C.D.: Translating the histone code. Science (New York, N.Y.) **293**(5532), 1074-1080 (2001). doi:10.1126/science.1063127
31. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., Zhang, Y.: Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science (New York, N.Y.) **298**(5595), 1039-1043 (2002). doi:10.1126/science.1076997
32. Walker, J.M.: The bicinchoninic acid (BCA) assay for protein quantitation. Methods in molecular biology (Clifton, N.J.) **32**(Journal Article), 5-8 (1994). doi:10.1385/0-89603-268-x:5
33. Pierantoni, G.M., Finelli, P., Valtorta, E., Giardino, D., Rodeschini, O., Esposito, F., Losa, M., Fusco, A., Larizza, L.: High-mobility group A2 gene expression is frequently induced in non-functioning pituitary adenomas (NFPAs), even in the absence of chromosome 12 polysomy. Endocr Relat Cancer **12**(4), 867-874 (2005). doi:10.1677/erc.1.01049
34. Wang, E.L., Qian, Z.R., Yamada, S., Rahman, M.M., Inosita, N., Kageji, T., Endo, H., Kudo, E., Sano, T.: Clinicopathological characterization of TSH-producing adenomas: special reference to TSH-immunoreactive but clinically non-functioning adenomas. Endocrine pathology **20**(4), 209-220 (2009). doi:10.1007/s12022-009-9094-y
35. De Martino, I., Visone, R., Fedele, M., Petrocca, F., Palmieri, D., Martinez Hoyos, J., Forzati, F., Croce, C.M., Fusco, A.: Regulation of microRNA expression by HMGA1 proteins. Oncogene **28**(11), 1432-1442 (2009). doi:10.1038/onc.2008.495
36. Baldassarre, G., Fedele, M., Battista, S., Vecchione, A., Klein-Szanto, A.J., Santoro, M., Waldmann, T.A., Azimi, N., Croce, C.M., Fusco, A.: Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway. Proc Natl Acad Sci U S A **98**(14), 7970-7975 (2001). doi:10.1073/pnas.141224998
37. Fedele, M., Battista, S., Kenyon, L., Baldassarre, G., Fidanza, V., Klein-Szanto, A.J., Parlow, A.F., Visone, R., Pierantoni, G.M., Outwater, E., Santoro, M., Croce, C.M., Fusco, A.: Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. Oncogene **21**(20), 3190-3198 (2002). doi:10.1038/sj.onc.1205428
38. Fedele, M., Pentimalli, F., Baldassarre, G., Battista, S., Klein-Szanto, A.J., Kenyon, L., Visone, R., De Martino, I., Ciarmiello, A., Arra, C., Viglietto, G., Croce, C.M., Fusco, A.: Transgenic mice overexpressing the wild-type form of the HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas. Oncogene **24**(21), 3427-3435 (2005). doi:10.1038/sj.onc.1208501
39. Fedele, M., Palmieri, D., Fusco, A.: HMGA2: A pituitary tumour subtype-specific oncogene? Molecular and cellular endocrinology **326**(1-2), 19-24 (2010). doi:10.1016/j.mce.2010.03.019
40. Butz, H., Liko, I., Czirjak, S., Igaz, P., Korbonits, M., Racz, K., Patocs, A.: MicroRNA profile indicates downregulation of the TGFbeta pathway in sporadic non-functioning pituitary adenomas. Pituitary **14**(2), 112-124 (2011). doi:10.1007/s11102-010-0268-x
41. Yacqub-Usman, K., Duong, C.V., Clayton, R.N., Farrell, W.E.: Preincubation of pituitary tumor cells with the epidrugs zebularine and trichostatin A are permissive for retinoic acid-augmented expression of the BMP-4 and D2R genes. Endocrinology **154**(5), 1711-1721 (2013). doi:10.1210/en.2013-1061

42. Lujambio, A., Ropero, S., Ballestar, E., Fraga, M.F., Cerrato, C., Setien, F., Casado, S., Suarez-Gauthier, A., Sanchez-Cespedes, M., Git, A., Spiteri, I., Das, P.P., Caldas, C., Miska, E., Esteller, M.: Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res **67**(4), 1424-1429 (2007). doi:10.1158/0008-5472.CAN-06-4218
43. Rhodes, L.V., Nitschke, A.M., Segar, H.C., Martin, E.C., Driver, J.L., Elliott, S., Nam, S.Y., Li, M., Nephew, K.P., Burow, M.E., Collins-Burow, B.M.: The histone deacetylase inhibitor trichostatin A alters microRNA expression profiles in apoptosis-resistant breast cancer cells. Oncol Rep **27**(1), 10-16 (2012). doi:10.3892/or.2011.1488
44. Sampath, D., Liu, C., Vasan, K., Sulda, M., Puduvalli, V.K., Wierda, W.G., Keating, M.J.: Histone deacetylases mediate the silencing of miR-15a, miR-16, and miR-29b in chronic lymphocytic leukemia. Blood **119**(5), 1162-1172 (2012). doi:10.1182/blood-2011-05-351510
45. Leone, V., Langella, C., D'Angelo, D., Mussnich, P., Wierinckx, A., Terracciano, L., Raverot, G., Lachuer, J., Rotondi, S., Jaffrain-Rea, M.L., Trouillas, J., Fusco, A.: miR-23b and miR-130b expression is downregulated in pituitary adenomas. Mol Cell Endocrinol **390**(1-2), 1-7 (2014). doi:10.1016/j.mce.2014.03.002
46. Bottoni, A., Zatelli, M.C., Ferracin, M., Tagliati, F., Piccin, D., Vignali, C., Calin, G.A., Negrini, M., Croce, C.M., Degli Uberti, E.C.: Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas. Journal of cellular physiology **210**(2), 370-377 (2007). doi:10.1002/jcp.20832
47. Mao, Z.G., He, D.S., Zhou, J., Yao, B., Xiao, W.W., Chen, C.H., Zhu, Y.H., Wang, H.J.: Differential expression of microRNAs in GH-secreting pituitary adenomas. Diagnostic pathology **5**, 79 (2010). doi:10.1186/1746-1596-5-79
48. Gadelha, M.R., Kasuki, L., Denes, J., Trivellin, G., Korbonits, M.: MicroRNAs: Suggested role in pituitary adenoma pathogenesis. Journal of endocrinological investigation **36**(10), 889-895 (2013).
49. Jiang, X., Zhang, X.: The Molecular Pathogenesis of Pituitary Adenomas: An Update. Endocrinology and metabolism **28**(4), 245-254 (2013). doi:10.3803/EnM.2013.28.4.245
50. Shi, X., Tao, B., He, H., Sun, Q., Fan, C., Bian, L., Zhao, W., Lu, Y.C.: MicroRNAs-based network: a novel therapeutic agent in pituitary adenoma. Medical hypotheses **78**(3), 380-384 (2012). doi:10.1016/j.mehy.2011.12.001
51. Rostad, S.: Pituitary adenoma pathogenesis: an update. Current opinion in endocrinology, diabetes, and obesity **19**(4), 322-327 (2012). doi:10.1097/MED.0b013e328354b2e2
52. Yacqub-Usman, K., Richardson, A., Duong, C.V., Clayton, R.N., Farrell, W.E.: The pituitary tumour epigenome: aberrations and prospects for targeted therapy. Nature reviews. Endocrinology **8**(8), 486-494 (2012). doi:10.1038/nrendo.2012.54

Figure 2


A
Relative transcript expression (%)
300
250
200
150
100
50
0
HMGA1
BMP-4
NRP
GH3
B
Relative transcript expression (%)
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100
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40
20
0
NRP
miR-16
miR-26b
miR-34b
miR-196ab
miR-let-7a

Fig 1

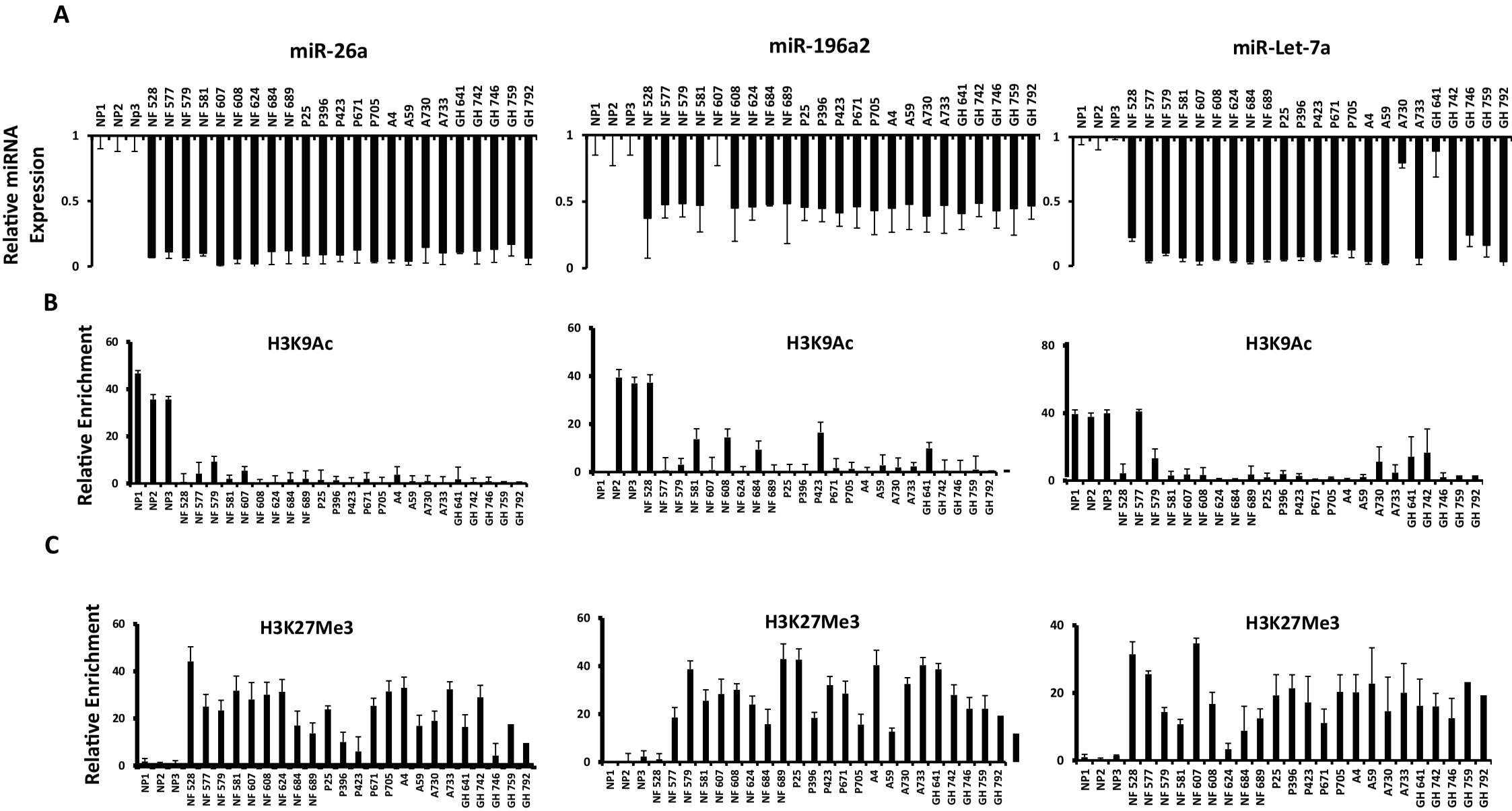

A
miR-26a
miR-196a2
miR-Let-7a
Relative miRNA Expression
NP1 NP2 NP3 NF 528 NF 577 NF 579 NF 581 NF 607 NF 608 NF 624 NF 684 NF 689 P25 P396 P423 P671 P705 A4 A59 A730 A733 GH 641 GH 742 GH 746 GH 759 GH 792
B
H3K9Ac
Relative Enrichment
C
H3K27Me3
Relative Enrichment

Figure 3

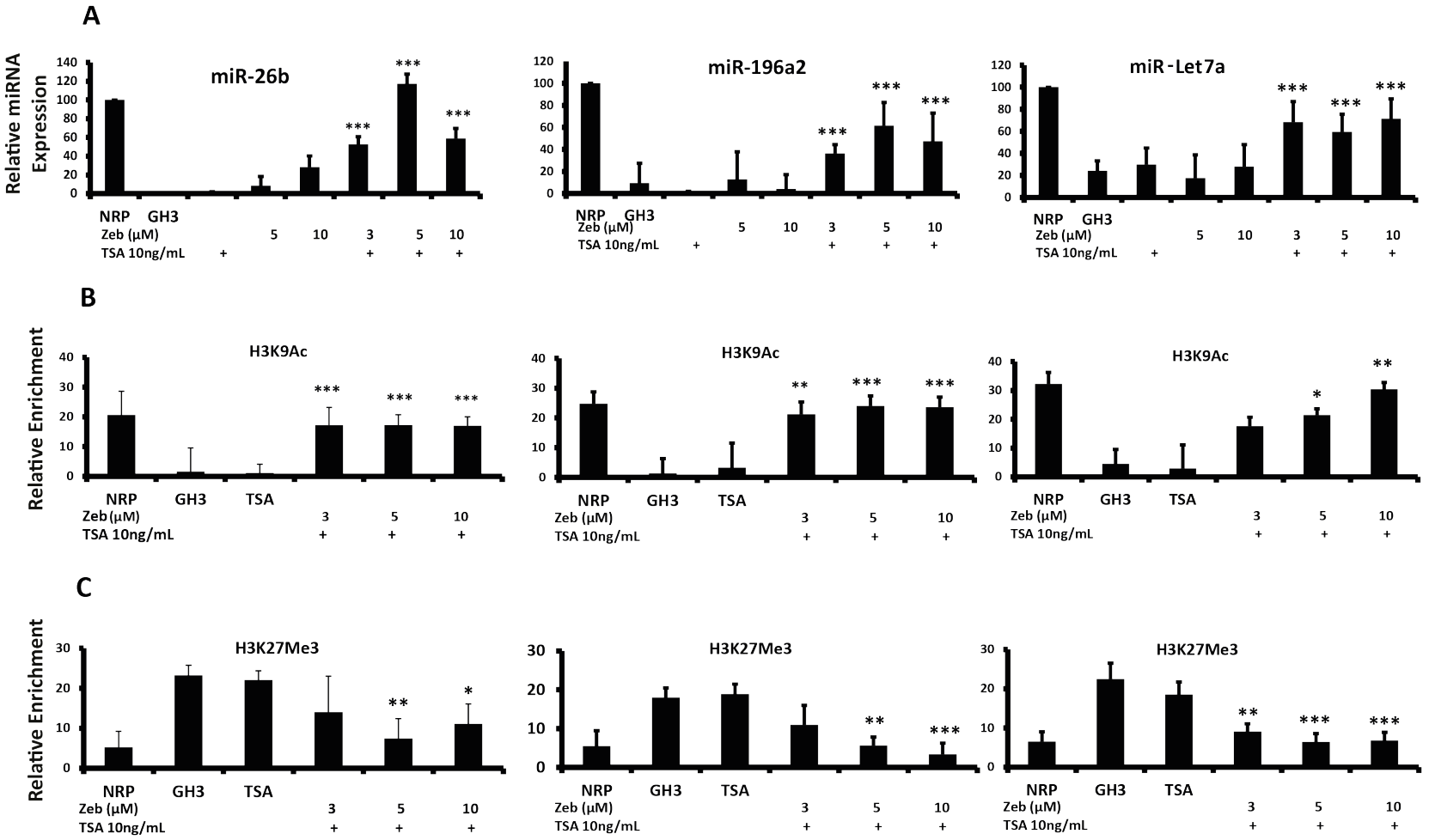
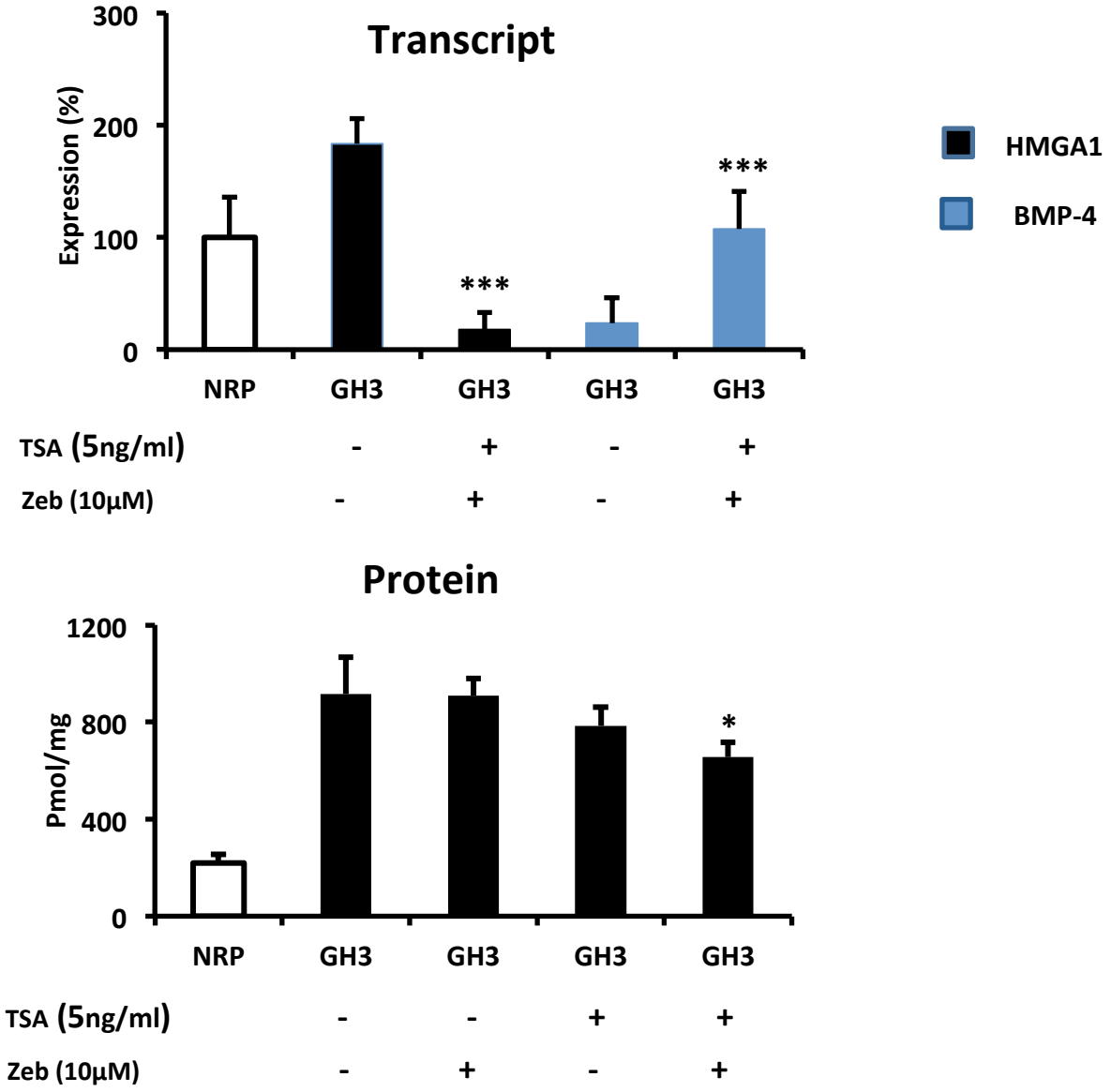

A
Relative miRNA Expression
miR-26b
miR-196a2
miR-Let7a
NRP GH3
Zeb (μM)
TSA 10ng/mL
B
Relative Enrichment
H3K9Ac
NRP GH3 TSA
C
H3K27Me3

Figure 4


Transcript
300
200
100
0
Expression (%)
***
***
HMGA1
BMP-4
NRP
GH3
GH3
GH3
GH3
TSA (5ng/ml)
- + - +
Zeb (10µM)
- + - +
Protein
1200
800
400
0
Pmol/mg
*
NRP
GH3
GH3
GH3
GH3
TSA (5ng/ml)
- - + +
Zeb (10µM)
- + - +