# Effect of 5-Aza-2´-Deoxycytidine and Trichostatin A on Endogenous *Versus* Ectopic Expression of Placental Members of the Human Growth Hormone Gene Family

By

Esha Ganguly

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Faculty of Health Sciences

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*“Truth can be stated in thousand different ways, yet each one can be true .....”* – Swami Vivekananda

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# ABSTRACT

***Background:*** The genes coding for human (*h*) chorionic somatomammotropin (CS), *hCS-A* and *hCS-B,* and placental growth hormone (GH-V), *hGH-V* are located at a single locus on chromosome 17q22-24. Local regulatory (5´ P and 3´ enhancer) sequences and a remote locus control region (LCR) containing a placenta-specific hypersensitive site (HS) IV, have been implicated in the efficient expression of the placental *h*CS/GH-V genes, in part through gene transfer studies in placental and non-placental tumor cell lines. However, low levels of endogenous expression are reported in placental tumor cells compared to normal term placenta. Thus it was ***hypothesized*** that the *hCS/GH-V* chromatin structure in human choriocarcinoma cells is less accessible to regulatory regions essential for efficient expression due to DNA and/or histone modifications, specifically methylation and acetylation, respectively.

***Approach:*** To assess individual *h*CS-A, *h*CS-B and *h*GH-V gene expression in placental and non-placental tumor cells, and assess the effect of increasing *"chromatin accessibility" on h*CS/GH-V RNA levels by inhibiting DNA methylation and histone deacetylation using 5-aza-2´-deoxycytidine (azadC) and trichostatin A (TSA).

***Principal Findings:*** Low levels of *h*CS-A, *h*CS-B and *h*GH-V RNA were detected in placental and non-placental tumor cells compared to term placenta. A significant >5-fold increase in promoter activity was seen in placental but not non-placental cells transfected with hybrid *h*CS promoter luciferase genes containing 3´-enhancer sequences. Placental JEG-3 cells pretreated with azadC and TSA resulted in a significant >10-fold increase in *h*CS-A, *h*CS-B and *h*GH-V RNA levels compared to TSA treatment alone, however, a modest ~3-fold effect was seen in

non-placental MCF-7 cells. By contrast to the effect of pretreatment with azadC, post-treatment with azadC mutes the stimulatory effects of TSA on *h*CS/GH-V transcripts. The specificity of the response suggests that azadC treatment, and presumably hypomethylation of DNA, results in an increase in response to TSA and histone hyperacetylation at the *h*GH/CS locus. An assessment of histone H3/H4 hyperacetylation in JEG-3 cells treated with azadC and TSA versus TSA alone revealed significant increases consistent with a more open chromatin structure including the *h*CS 3´-enhancer sequences and LCR.

***Conclusions:*** These observations suggest that accessibility of remote and local regulatory regions required for efficient placental *h*GH/CS expression can be restricted by DNA methylation and histone acetylation status. This includes restricting access of the *h*CS 3´-enhancer sequences to available placental enhancer transcription factors.

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# ABBREVIATIONS

| | |
|---|---|
| 5- mC | 5- methyl cytosine |
| ANOVA | analysis of variance |
| AP2 | activating protein 2 |
| bp | base pair |
| CBP | creb-binding protein |
| C/EBP | ccaat-enhancer-binding proteins |
| CG | chorionic gonadotropin |
| ChIP | chromatin immunoprecipitation |
| CS-A/-B/-L | chorionic somatomammotropin-A/-B/-like |
| CSp | chorionic somatomammotropin promoter |
| CTCF | CCCTC binding-factor |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | dimethyl sulfoxide |
| DNase | deoxyribonuclease |

| | |
|---|---|
| DNMT | DNA methyl-transferase |
| ECL | enhanced chemiluminescence |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| Enh | enhancer |
| FOXF1 | forkhead box F1 |
| GAPDH | glyceraldehyde-3-dehydrogenase |
| *h* | human |
| HAc | hyperacetylated |
| HAT | histone acetyl-transferase |
| HDAC | histone de-acetylase |
| HMT | histone methyl-transferase |
| HP1 | heterochromatin protein 1 |
| HS | hypersensitive |
| IgG | immunoglobulin G |
| kb | kilobases |

| | |
|---|---|
| LHR | luteinizing hormone receptor |
| Luc | luciferase |
| µM | micro molar |
| MBD | methyl binding domain |
| MCF-7 | Michigan Cancer Foundation-7 |
| MECP2 | methyl-CpG-binding protein 2 |
| mRNA | messenger ribonucleic acid |
| ng | nanogram |
| NuRD | nucleosome remodeling and histone deacetylases complex |
| OCT4 | octamer-binding transcription factor 4 |
| PCR | Polymerase Chain Reaction |
| q-PCR | quantitative polymerase chain reaction |
| R-Luc | *renilla*-luciferase |
| RNA | ribonucleic acid |
| RPMI | Rosewell Park Memorial Institute |

| | |
|---|---|
| Sp1 | specificity protein 1 |
| SW1/SNF | SWItch/sucrose non-fermentable |

# CHAPTER 1

# Introduction

## *1.1 Regulation of Differential Gene Expression Pattern*

Regulation of gene expression in higher eukaryotes is a complex and tightly regulated process involving many factors and levels of control (Barrett, Fletcher et al. 2012). Expression of individual genes from somatic cell genome, varies both temporally, that is at certain stage of development or in response to signals, as well as spatially, where expression occurs in one cell type and not the other. Recent evidence suggests that regulatory mechanisms underlying gene expression are becoming increasingly well understood (Tyagi 2015).

Gene regulation is a highly dynamic process that includes two stages. The first stage involves the structural constraints that determine accessibility of genes, and the second stage involves the initiation of transcription that occurs once the gene is accessible.

## *1.2 Chromatin Structure*

The structure of chromatin not only helps package the entire eukaryotic genome into the nucleus, but also helps determine the accessibility of DNA for replication and selective transcription. *"Nucleosomes"* are the repeating subunits of chromatin (Luger, Dechassa et al. 2012). They consist of a core of basic histone proteins, around which the DNA is wrapped 1.65 times. The core is an octamer that consist of two copies of each of the canonical histones: H3, H4, H2A and

H2B (Kornberg 1974).

Histone proteins H3, H4, H2A and H2B, share a common structural domain that consist of three-α helices (α1, α2 and α3) separated by two loops (L1 and L2), also known as the *histone fold*, which facilitate heterodimerization of H2A with H2B and H3 with H4 (Luger, Mader et al. 1997). Such interactions are responsible for the basic dimeric structural unit of the nucleosome (Figure 1.1).

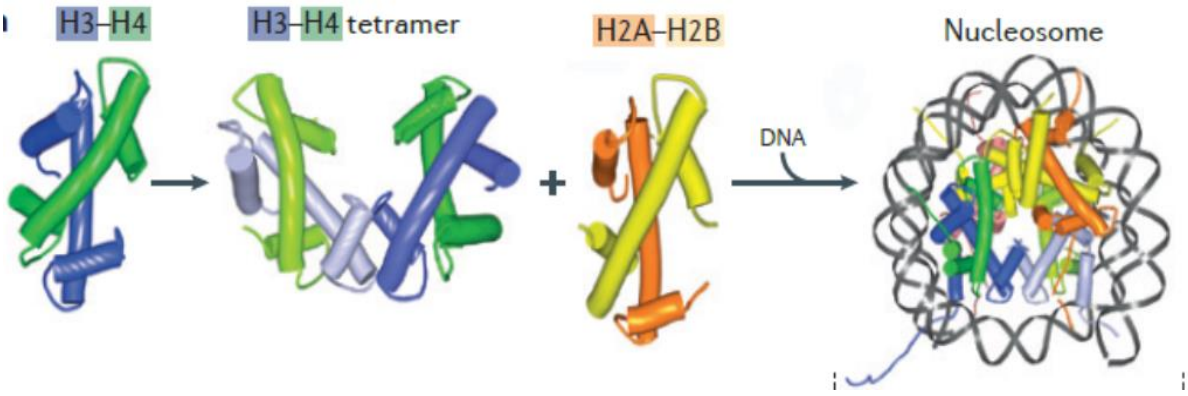



***Figure 1.1: The nucleosome: basic repetitive unit of chromatin***

The above figures represent structural elements that help to maintain the nucleosome. It consists of the two-histone fold domain formed by three α-helices separated by loops. Two H3-H4 heterodimers form a four-helix bundle-giving rise to a tetramer. Two H2A-H2B then associate with H4 to form an octamer, which forms a nucleosome. This structure represents a hierarchical arrangement of histones in the nucleosome. Figure 1.1 is reproduced from (Venkatesh and Workman 2015).

The N- and C-terminal tails protrude from the globular portion of histone H2A, H2B, H3 and H4 and can undergo several types of post-translational modifications (Table. 1) (Kouzarides 2007). Histones were originally considered as a static scaffold for DNA packaging (Zhou, Goren et al.

2011). However, it is now known that the post-translational modifications of the histone tails dictate DNA packaging which can modulate its accessibility (Felsenfeld and Groudine 2003, Zhou, Goren et al. 2011).

Different patterns of post-translational modifications established on distinct amino acid residues on histone proteins can regulate two opposite processes like transcriptional activation and repression (Table 1). Additionally, post-translationally modified chromatin also serves as selective "*binding platform"* for regulatory proteins that can specifically bind to certain modified amino acid residues and mediate the initiation of nuclear processes such as transcription, DNA repair and replication (Kouzarides 2007, Taverna, Li et al. 2007).

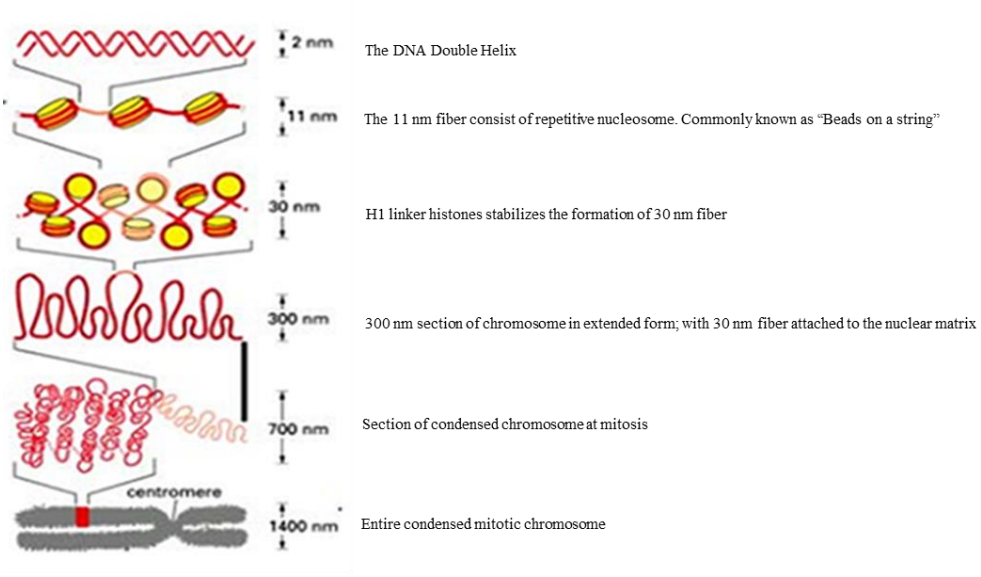



***Figure 1.2: Packaging of Chromatin in the Nucleus***:

This schematic represents the different levels of chromatin compaction in the eukaryotic nucleus from "naked" DNA to the condensed mitotic chromosome. This schematic representation is modified from Alberts and Watson (1994).

### *1.3 Genome accessibility and activation of gene expression*

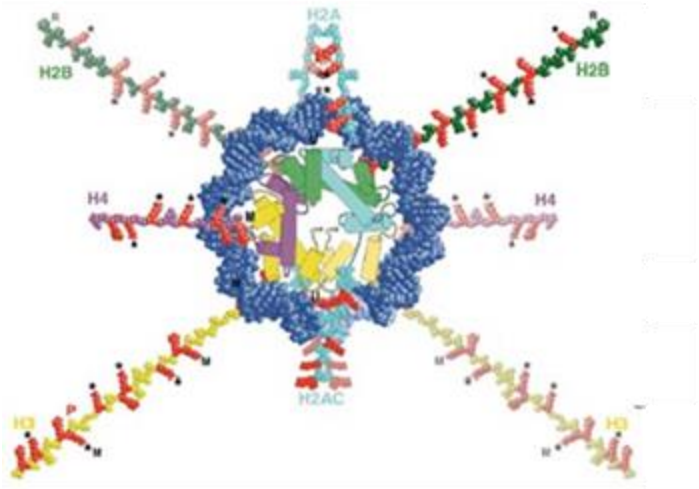
*DNA accessibility* is indirectly determined by preferential catalytic activity of DNase I –*"an enzyme that preferentially snips accessible"* DNA (Casci 2006). The digestion sites referred to as *"DNase I hypersensitive sites"* usually reside in the *cis*-regulatory elements such as promoters and enhancers of actively transcribed genes (Bell, Tiwari et al. 2011). Classically, there are two major classes of morphologically distinct chromatin in eukaryotes: (a) *Heterochromatin* that is compact and transcriptionally inactive and (b) *Euchromatin* that is less compact and more transcriptionally active (Hsu 1962, Frenster, Allfrey et al. 1963). Chromatin structure changes include unwrapping, rewrapping, sliding, assembly and disassembly of nucleosomes, which involve the formation and disruption of interactions between DNA, H3-H4 and H2A-H2B components of the nucleosome (Zhang and Tang 2003, Mersfelder and Parthun 2006, Arnaudo and Garcia 2013). These chromatin structure modifications are influenced by post-translational modifications of histones (Zhang and Tang 2003, Mersfelder and Parthun 2006, Arnaudo and Garcia 2013).

As discussed in Section *1.2* post-translational modifications of histones impact chromatin structure by influencing histone-DNA and histone-histone contacts (Wolffe 1998, Wolffe and Hayes 1999). The presence of these marks at the protruding N-terminal tails are viewed as acting in two ways: a) *directly*, by altering the structure of local chromatin to open and restrict access to DNA; and b) *indirectly*, through the recruitment of effector proteins that carry out transcription and other nuclear processes (Strahl and Allis 2000, Jenuwein and Allis 2001, Gurard-Levin and Almouzni 2014). The latter led to the *"Histone Code Hypothesis"*, which states that post-translational modifications on one or multiple amino acid residue of histone tails may act

sequentially or in combination to specify downstream signal (Strahl and Allis 2000). For example: phosphorylation of serine 10 residue on histone H3 is associated with chromosome condensation during mitosis and early gene induction following mitogenic stimulation (Strahl and Allis 2000). A review suggested that a phosphorylation mark alone or in combination with other marks (such as phosphorylation at serine 28 residue on histone H3) may recruit factors that mediate chromosome condensation and segregation (Strahl and Allis 2000, Rothbart and Strahl 2014).

***Table 1*:** List of post-translational modification. This table is modified from (Sadakierska-Chudy and Filip 2015).

| **Amino acid residue [single-letter code]** | **MODIFICATION** |
|---|---|
| **Lysine [K]** | Acetylation |
| | Mono-, di-, and trimethylation |
| | Ubiquitination |
| | Biotinylation |
| | Sumoylation |
| **Arginine [R]** | Mono- and di- methylation |
| | Mono-and poly-ADP-ribosylation |
| **Serine [S]** | Phosphorylation |
| **Tyrosine [Y]** | Phosphorylation |
| **Threonine [T]** | Phosphorylation |




Tails comprise 25 – 30% of histone mass.

Tails are rich in:

a) Lysine (K): Acetylation
Methylation
Ubiquitination

b) Serine (S): Phosphorylation

c) Arginine (R): Methylation

***Figure 1.3: Representative Image of Histone Tails and Associated Modifications***

Acetylation of lysine is a major post-translational modification. Histone acetylation can influence the occurrence of other posttranslational modifications such as methylation, ubiquitination and phosphorylation either in *cis*, on the same histone molecule, or in *trans*, between histone molecules or across nucleosomes (Kouzarides 2007, Latham and Dent 2007). One of the best studied example of cross-regulation is phosphorylation of histone H3 at Ser10 followed shortly by acetylation at Lys14 in mouse 10 T1/2 cells upon stimulation of the Ras-MAPK pathway with epidermal growth factor (EGF) (Hsu, Sun et al. 2000, Lo, Trievel et al. 2000, Liokatis, Stutzer et al. 2012). Acetylation is now known to occur in more than 80 transcription factors and many other cytoplasmic proteins (Glozak, Sengupta et al. 2005). Thus, it is not only crucial in the nucleus but also for regulating different cytoplasmic process including energy metabolism,

autophagy, endocytosis and even signaling pathways from the plasma membrane (Matthias, Yoshida et al. 2008, Yang and Seto 2008, Sigismund, Confalonieri et al. 2012).

### *1.4 Histone Acetylation*

Acetylation of histones H3 and H4 makes the chromatin more accessible to interacting proteins *in vivo* (Krajewski and Becker 1998). *In vitro* demonstrations have also illustrated that acetylation counteracts the tendency of nucleosomes to fold into highly compact inaccessible structures (Hebbes, Clayton et al. 1994, Garcia-Ramirez, Rocchini et al. 1995, Krajewski and Becker 1998, Tse, Sera et al. 1998). In eukaryotic cells, most of the genome consists of hypoacetylated inactive chromatin usually considered as the *"ground state"* (Schubeler, Francastel et al. 2000). Activation of genes requires hyperacetylation of the histones across the chromatin domain. The well-defined β-globin locus reveals that *"transcriptional competence"* require acetylation throughout the majority of the domain (Litt, Simpson et al. 2001, Schubeler, Groudine et al. 2001).

Acetylation of H3-H4 histones across chromatin regions is correlated with *transcriptional competence,* decondensation of the chromatin and renders the region *permissive* to transcriptional factors (Schubeler, Francastel et al. 2000). Studies have demonstrated that transcriptional activation occurs within a *permissive* promoter region marked by localized and targeted acetylation (Brown, Lechner et al. 2000, Forsberg and Bresnick 2001). A more localized acetylation of histone H3 works in conjunction with broader range of histone H4 acetylation (Schubeler, Francastel et al. 2000, Vignali, Steger et al. 2000). Regions located near the

transcription initiation site (Promoters) and *cis* regulatory regions hundreds of base pairs away from the initiation site (Enhancers) are enriched with hyperacetylated histones (Litt, Simpson et al. 2001). Hyperacetylated histones are also found at DNase I hypersensitive sites (Litt, Simpson et al. 2001). As discussed in Section *1.3*, acetylation facilitates recruitment of proteins to the exposed DNA. Transcriptional regulation involves targeting of acetylation enzymes HATS (co-activators of transcription) and deacetylation enzymes HDACs (repressors of transcription) to regulatory sites and these trans-factors recruit HAT containing-activators to specific promoters or enhancer regions (Brown, Lechner et al. 2000). Possible mechanisms proposed for tethering of HATs to permissive chromatin regions include the recruitment of HATs to *"distinct entry sites from where they spread throughout the domain"* (Kelley and Kuroda 2000, Koch and Andrau 2011, Varala, Li et al. 2015) possibly by attaching to tracking protein such as RNA polymerase II (Wittschieben, Otero et al. 1999).

A permissive chromatin structure by domain-wide histone acetylation is necessary but not sufficient for active transcription (Struhl 1998). In the event of an unfolded and more accessible chromatin structure, a single nucleosome is considered to be an obstacle to the interactions between transcription factors and their binding counterparts (Struhl 1998). A hyperacetylated nucleosome is less restrictive (Vettese-Dadey, Grant et al. 1996, Anderson, Lowary et al. 2001, Sewack, Ellis et al. 2001) and increases the flexibility of DNA ends (Eberharter and Becker 2002). As mentioned in Section *1.3*, changes to the chromatin structure that accompany transcriptional activation requires not only acetylation alone but the combined actions of several other post-translational modifications (Wang, Moore et al. 2000).

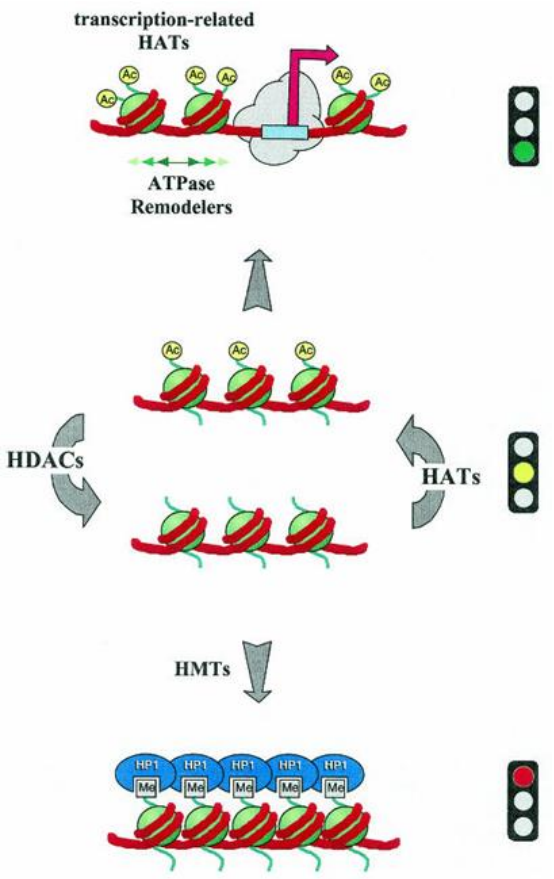



***Figure 1.4: Histone Acetylation Switch***

Acetylation permits the recruitment of ATP-dependent chromatin remodeling factors to open the promoters and regulatory regions. Deacetylation by HDACs; often followed by histone methylation lead to heterochromatin which is a highly repressive structure. In this illustration: Acetylated histone tail is indicated as yellow circles. Methylation is indicated by grey rectangle. HAT – Histone AcetylTransferase; HDAC – Histone DeACetylase; HMT – Histone MethylTransferase; HP1 – Heterochromatin Protein 1. This Figure is reproduced from (Eberharter and Becker 2002).

### *1.5 DNA Methylation*

Methylation of the cytosine residues in the context of CpG dinucleotide serve as a *"silencing"* mark in vertebrates (Holliday and Pugh 1975, Riggs 1975). More than 50% of the genes in the vertebrate's genome consist of an approximately 1 kb short CpG rich region also known as *CpG islands*. CpG regions in a mammalian genome are mostly concentrated in the promoter region but are also distributed across the genome (Jones 1999, Takai and Jones 2002).

CpG islands have a bimodal pattern of distribution (Takai and Jones 2002). The *de novo* methyltransferases, DNMT3A and DNMT3B are responsible for setting up the methylation pattern during development. The substrates for these enzymes include the nucleosomal DNA. (Ooi, Qiu et al. 2007). Following replication, the methylation pattern is maintained by maintenance methyltransferase DNMT 1 (Jones and Liang 2009).

A transcriptionally active gene is characterized by nucleosome depleted regions and the level of gene expression in eukaryotes is controlled by transcription factors (Kelly, Miranda et al. 2010). Nucleosomes are considered to be preferential sites for DNA methylation (Chodavarapu, Feng et al. 2010). There is evidence which suggests that methylated DNA at the transcription start site cannot initiate transcription as the chromatin is folded into higher-order structure (Kass, Landsberger et al. 1997, Zhang and Tang 2003, Hernando-Herraez, Heyn et al. 2015). Methylation sites at gene promoters are associated with repressed transcription and marked by methyl-CpG-binding protein 2 (MECP2) which recruits HDACs (repressors of transcription) (Nguyen, Gonzales et al. 2001).

***In summary, DNA methylation is considered to be a "silencing" mark, which represses***

***transcription.***

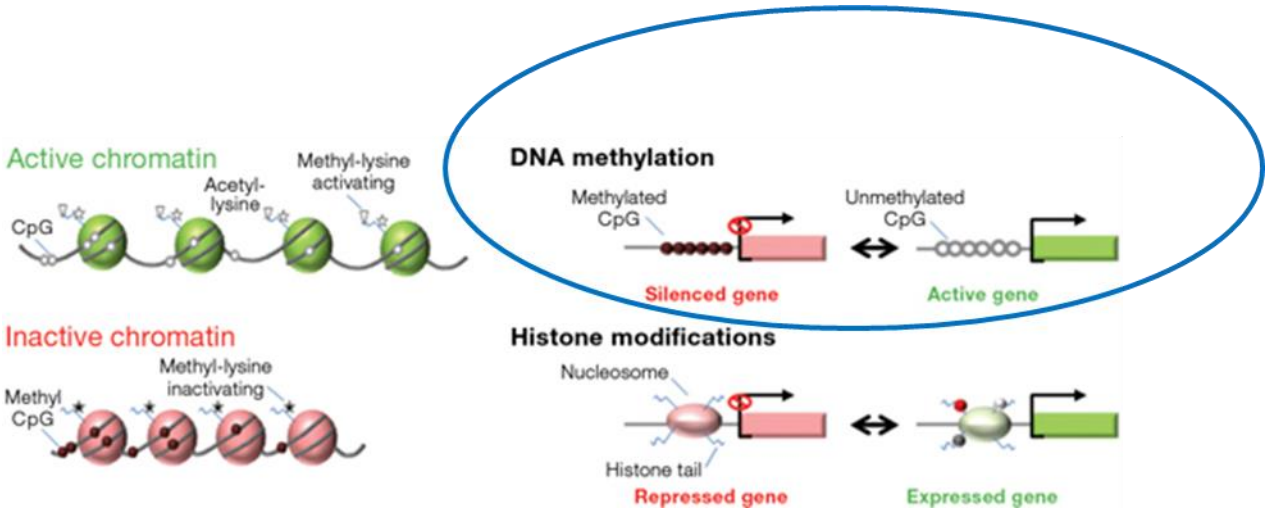



***Figure 1.5: DNA Methylation***

Methylation of cytosine in CpG dinucleotide is associated with condensed, inactive chromatin. The repressive state is maintained by lysine modifications of the histone tails. DNA methylation involves addition of methyl group to the 5-cytosine residues in CpG dinucleotide residues. This Figure is reproduced from (Gudjonsson and Krueger 2012).

### *1.6 The Human Growth Hormone (GH) / Chorionic Somatomammotropin (CS) gene family as a model system to study differential gene expression*

The human GH/CS gene locus on chromosome 17 contains five tandemly arranged and structurally highly related genes. From 5´ to 3´, the genes within the locus are the pituitary growth hormone *hGH-N* and the placental *hCS-L*, *hCS-A*, *hGH-V*, and *hCS-B*.

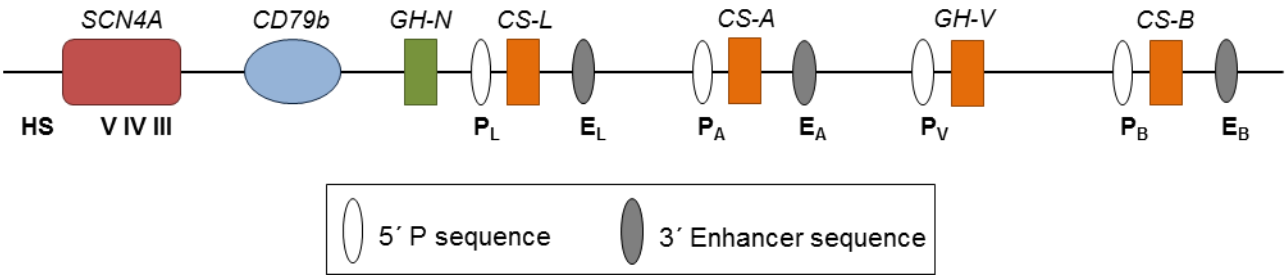



***Figure 1.6: The Human (h) Growth Hormone (GH) – Chorionic Somatomammotropin (CS) Locus***

From 5´ to 3´ the genes within the locus are Growth Hormone-N (*GH-N*), Chorionic Somatomammotropin-L (*CS-L*), Chorionic Somatomammotropin-A (*CS-A*), Growth Hormone Variant (*GH-V*), and Chorionic Somatomammotropin-B (*CS-B*).

These genes are believed to have evolved through gene duplication, resulting in greater than 90% similarity in gene structures and flanking regulatory sequences (Barsh, Seeburg et al. 1983, Hirt, Kimelman et al. 1987, Chen, Liao et al. 1989). *Human* GH-N is primarily expressed in the

somatotrophs of the anterior pituitary and *h*CS and *h*GH-V genes are expressed in the placental syncytiotrophoblasts (Chen, Liao et al. 1989). The *h*GH-V gene codes for placental GH and *hCS-A* and *hCS-B* independently code for the same polypeptide hormone *h*CS (also known as placental lactogen), while *hCS-L* is a pesudogene (Liebhaber, Urbanek et al. 1989, MacLeod, Lee et al. 1992).

### *1.6.1 Human term placenta*

Levels of *h*CS and *h*GH-V correlate with placental development and mass during pregnancy. These pregnancy-specific hormones may help to control insulin sensitivity in normal pregnancies through positive effects on pancreatic β cell mass (Hoshina, Boothby et al. 1982, Lombardo, De Angelis et al. 2011). The expression of *h*CS in normal placenta represents 3.5% of the total mRNA of syncytiotrophoblasts, which is similar to the 3% of total GH-N mRNA represented in the pituitary somatotrophs. By contrast, *h*GH-V transcripts account for closer to 0.001% of the total syncytiotrophoblast mRNA (Liebhaber, Urbanek et al. 1989, MacLeod, Lee et al. 1992). Expression of *h*CS is high during pregnancy (grams of protein per day in the latter stages) and it has been reported that the *h*CS genes are differentially expressed during pregnancy (MacLeod, Lee et al. 1992). *Human* CS-A and *h*CS-B RNA levels are comparable at eight weeks of pregnancy but *h*CS-A transcripts are five-fold more abundantly expressed than *h*CS-B by term (MacLeod, Lee et al. 1992). There is also evidence that *h*CS/GH-V RNA levels are decreased in pregnancies complicated by maternal obesity (Vakili, Jin et al. 2013).

### *1.6.2 Human choriocarcinoma cell lines*

Human placental choriocarcinoma cell lines (JEG-3, JAR and BeWo) consist of *"mitotically*

*active cytotrophoblasts with moderate degrees of differentiation"* (Ringler and Strauss 1990). Human placental choriocarcinoma cell lines, including JEG-3, JAR and BeWo express the *h*CS/GH-V genes at much lower levels relative to human term placenta (Nickel and Cattini 1991, Lytras, Bock et al. 1994). The nuclease sensitivity assay pattern is suggestive of a more open chromatin configuration of the *h*CS/GH-V locus in term placenta (Nickel and Cattini 1996). Thus, although transcript levels are low, human choriocarcinoma cell lines have offered a model to study *h*CS/GH-V gene regulation.

### *1.6.3 Ectopic expression*

Studies have also revealed ectopic *h*CS/GH-V gene expression in human endometrial tissue (Slater, Cooper et al. 2006), normal human mammary gland (Mol, Henzen-Logmans et al. 1995, Raccurt, Lobie et al. 2002), normal and neoplastic human lymphoid tissues and cells (Hattori, Shimatsu et al. 1990, Wu, Devi et al. 1996), human ovaries (Untergasser, Kranewitter et al. 1997, Silva, Figueiredo et al. 2009), human testis (Untergasser, Kranewitter et al. 1997) and also in non-placental endometrial carcinoma cell lines (Khorram, Garthwaite et al. 2001, Sbracia, Scarpellini et al. 2004, Pandey, Perry et al. 2008), adenocarcinoma cells such as the breast cancer MCF-7 cell line (Tuttle, Hugo et al. 2014). However, the expression profile of the individual members of the *h*CS/GH-V gene family is not known.

## *1.7 Human (h) CS/GH locus is a target for histone and/or DNA modifications and regulatory regions linked to expression of the placental members of the human (h) GH/CS locus*

As mentioned in Section *1.6.1*, *h*CS is expressed at high levels during normal pregnancy. Expression of the *h*CS/GH-V genes in placental cells has been linked to multiple regulatory

regions based on gene transfer studies in culture and mouse *in vivo*. As discussed in detail in Section *4.1*, enhancer activity was localized within a 241 bp fragment of a larger 1022 bp region downstream of the *h*CS-B gene, which has the capacity to increase CS promoter activity in transiently transfected placental cell lines by 300 fold (Jacquemin, Oury et al. 1994, Lytras and Cattini 1994). The *h*CS enhancer sequence is a repeat sequence and is also present downstream of *h*CS-A and *h*CS-L genes (Fitzpatrick, Walker et al. 1990). The *h*CS-A and *h*CS-L enhancers are not as potent as the *h*CS-B enhancer in transfected choriocarcinoma cells although, the activity of *h*CS-A enhancer sequence increases in placental primary cells undergoing differentiation (Jacquemin, Alsat et al. 1996). These observations have suggested that the *h*CS enhancers might contribute to activated expression of *hCS* in normal term placenta *in vivo*.

The locus control region (LCR) for the *h*CS/GH-V gene locus has three hypersensitive sites (HS) known to be involved in regulation of the placental genes. HS III and HS V regions were identified in term placenta as well as pituitary adenoma chromatin, but HS IV was specific to the placenta (Jones, Monks et al. 1995). Using ChIP assays hyperacetylation of histones in the HS III and HS V regions has been observed. This study also demonstrated hyperacetylation of the proximal upstream P sequences (Elefant, Su et al. 2000); P sequences were first identified as repeated regions found in the *h*GH/CS gene locus on chromosome 17 and located about 2 kb upstream of each of the placental members (Barsh, Seeburg et al. 1983, Chen, Liao et al. 1989) There is evidence that perhaps independently none of the regulatory regions are sufficient to confer efficient and consistent expression of the *h*CS/GH-V genes in the placenta. P sequences are conserved sequences located approximately 2 kb upstream of *h*CS-A, *h*CS-B and *h*GH-V genes (Nachtigal, Nickel et al. 1993). P sequence has the capacity to enhance transgene activity

in the placenta of transgenic mice (Elefant, Su et al. 2000). Placental transgene expression was evident in two of the three HSI,II P-*h*GH-N-P compared to similar construct which lacked the 263P element (Elefant, Su et al. 2000). However, a 15 kb *h*CS-A transgene containing enough flanking DNA to include upstream P sequence and a downstream enhancer region was expressed in only 4 of the 6 transgenic lines (Jones, Monks et al. 1995). In each of these constructs, the level of transgene expression was variable (Elefant, Su et al. 2000). It can therefore be interpreted that the distal HS III-V which was not contained in HSI,II P-*h*GH-N-P construct, has the capacity to produce expression level (Elefant, Su et al. 2000). It is still unclear if the P sequence can act as a local enhancer, but it would be consistent with the observation of P sequence hyperacetylation in human term placenta (Elefant, Su et al. 2000). There is a more likely possibility that the P sequences act in conjunction with other regions not limited to promoters and downstream enhancers for activated expression. The single construct that has been reported to confer consistent expression of the locus in the placenta includes the entire *h*GH/CS gene locus and LCR (Jin, Lu et al. 2009). Taken together these results suggest that neither proximal nor distal regulatory elements alone are sufficient for the appropriate expression of *h*GH/CS gene family in the placenta.

Information contained in Sections ***1.1-1.7*** of the thesis, suggest that histone acetylation and DNA methylation can contribute to accessibility of the chromatin. High levels of *h*CS/GH-V in human term placenta indicate a more 'open' and accessible *h*GH/CS locus in human term placenta *versus* choriocarcinoma cells. Several proximal and distal regulatory regions have been linked to efficient expression of the *h*CS/GH-V genes as implied above. Neither the proximal nor distal elements alone are, however, sufficient for efficient and appropriate expression of the

placental members of the *h*CS/GH locus (Jones, Monks et al. 1995). There is a possibility that the regulatory regions need to be accessible to facilitate co-operation with each other for activated expression.

### *1.8 Overview of Thesis Research Objectives*

The human (*h*) chorionic somatomammotropin (CS) genes (*hCS-A* and *hCS-B* as well as the *hCS-L* pseudogene) and placental growth hormone (GH) variant gene (*hGH-V*) are located at a single locus on chromosome 17q22-24 (Chen, Liao et al. 1989). These genes are preferentially expressed by villus syncytiotrophoblasts in the placenta during pregnancy (Liebhaber, Urbanek et al. 1989, MacLeod, Lee et al. 1992). There is also evidence that these genes are differentially expressed; *h*CS-A and *h*CS-B RNA levels are comparable at eight weeks of pregnancy, but *h*CS-A transcripts are five-fold more abundant than those of *h*CS-B by term (MacLeod, Lee et al. 1992). Human CS genes are expressed at relatively high levels such that *h*CS RNA represent about 3.5% of the total syncytiotrophoblast transcripts, and are also more abundant than those of *h*GH-V in term placenta (Cooke, Ray et al. 1988, MacLeod, Lee et al. 1992). These patterns of *h*CS/GH-V gene expression are not always conserved in placental tissues or cells. For example, *h*CS/GH-V RNA levels are decreased in pregnancies complicated by obesity (Vakili, Jin et al. 2013) or altered in abnormal placental tissue or cells, including hydatidiform mole and choriocarcinoma cell line (Nickel and Cattini 1991). Relatively low levels of *h*CS/GH-V transcripts were detected in human choriocarcinoma BeWo, JAR and JEG-3 cells compared to normal term placenta (Lytras, Bock et al. 1994). In addition, there is evidence for low-level ectopic production of *h*CS/GH-V transcripts in ovaries, testis, mammary glands, and endometrium, and also in some non-placental tumor cells such as the breast cancer Michigan Cancer Foundation-7 (MCF-7) cell line (Tuttle, Hugo et al. 2014).

Based on *in vitro* and transgenic mouse *in vivo* study, multiple ***regulatory regions*** are implicated in the efficient expression of the *h*CS/GH-V genes (Jones, Monks et al. 1995). These include the

proximal promoter region and P sequences located about 2 kb upstream, as well as 3´-enhancer regions located about 2 kb downstream of the *h*CS genes (Chen, Liao et al. 1989, Jones, Monks et al. 1995). In addition, remote upstream nuclease hypersensitive sites (HS) have been identified in a LCR including HS III and HS V, and the placenta-specific HS-IV (Chen, Liao et al. 1989, Nachtigal, Nickel et al. 1993, Jones, Monks et al. 1995, Elefant, Su et al. 2000, Lytras, Detillieux et al. 2011).

Thus, it is ***hypothesized*** that low levels of *h*CS/GH-V expression in placental tumor cells is associated with DNA (methylation) and/or histone (acetylation) modifications, which limits accessibility of necessary transcription factors to specific regulatory regions. Furthermore, it is ***hypothesized*** that ectopic expression may be associated with a different pattern of DNA (methylation) and/or histone (acetylation) modification, likely reflecting a distinct chromatin structure or pattern of regulatory regions and/or associated transcription factors, even when overall levels of *h*CS/GH-V RNA are comparable to those in placental cells. Four research ***objectives*** are pursued in this thesis. They are:

1. ***To confirm and compare hGH/CS expression levels in tumor cells of placental and non-placental origin versus normal human term placenta (Chapter 3).***

   ***Approach:*** In brief, total *h*CS/GH-V RNA levels will be compared in human placental (BeWo, JAR and JEG-3) and non-placental cervical carcinoma HeLa, breast adenocarcinoma MCF-7 and T47D, uterine adenocarcinoma Hec1A and glioblastoma U-87

cells. RNA from HTP and cell lines will be assessed by reverse transcriptase (RT) polymerase chain reaction (PCR). A set of exon 3/exon 4 primers common to all members of the hCS/GH-V gene family will be used to non-selectively amplify the known majority of *h*GH/CS transcripts (which can be easily distinguished from unprocessed message or DNA).

**2.** ***To compare individual hCS-A, hCS-B and hGH-V RNA levels in tumor cells of placental versus non-placental origin (Chapter 3).***

***Approach:*** In brief, quantitative real-time RT-PCR (q-PCR) will be combined with specific primers to detect *h*CS-A, versus *h*CS-B versus *h*GH-V transcripts. Cells to be tested will be based on the results of *Objective 1*, but will include tumor cells showing the *highest* and *lowest* hCS-GH/V RNA levels, and if possible an example of placental and non-placental cells with comparable expression levels.

**3.** To compare *h*CS promoter, P sequence and 3´-enhancer activity in transfected placental and non-placental tumor cells as an indication of transcription factor availability. Ideally an example of each cell type will be selected where both display comparable levels of *h*CS/GH-V transcripts as detected by RT-PCR **(Chapter 4)**.

***Approach:*** In brief, placental and non-placental cells will be transiently transfected with hybrid luciferase (Luc) genes driven by a 496 bp *h*CS-A promoter region with or without a 263 bp P sequence or 241 bp *h*CS-B 3´-enhancer regions. Cells will also be transfected with luciferase genes driven by a minimal (84 bp viral) thymidine kinase and 496 bp pituitary *h*GH promoter as well a promoter-less gene as controls. All Luc gene constructs will be co-transfected with a Renilla-luciferase (R-Luc) gene and relative luciferase activity (Luc/R-Luc) determined for comparison.

4. ***To compare the effect of histone acetylation and/or DNA methylation inhibition on hCS/GH-V gene expression in placental and non-placental cells, as a means to increase transcription factor accessibility. Ideally an example of each cell type will be selected where both display comparable levels of hCS/GH-V transcripts as detected by RT-PCR (Chapter 5).***

***Approach:*** In brief, placental and non-placental tumor cells will be treated with increasing doses of the histone deacetylases (HDAC) inhibitor trichostatin-A (TSA) and/or the DNA methyltransferase (DNMT1) inhibitor 5-aza-2´ deoxycytidine (azadC) at increasing doses for 18 or 24 hours. RNA levels will be assessed for *h*CS-A, *h*CS-B and *h*GH-V by q-PCR. Effects on histone acetylation and DNA methylation will be confirmed by protein "immuno"blotting using specific antibodies to hyperacetylated histones and a commercially available enzyme-linked immune-sorbent assay (ELISA) to detect methylated DNA. If there

is a significant effect of these treatments on *h*CS/GH-V transcript levels then an attempt will be made to link them to any corresponding changes in known regulatory region structure associated with *h*CS/GH-V gene expression, including, promoter, P and 3´-enhancer sequences.

The observations made will be discussed in the context of how differences in the organization or structure of the *h*CS/GH-V gene locus on chromosome 17 can affect both endogenous and ectopic *h*CS/GH-V RNA expression, and whether this (i) may have broader implications, and (ii) how this might be pursued.

# CHAPTER 2

## Materials and Methods

### *2.1 Human Term Placenta Samples*

Human term placenta (HTP) samples were obtained after approval of the Health Research Ethics Board at the University of Manitoba. Placentas were obtained from women with a body mass index (BMI) of 20-25 $kg/m^{2,}$ and no gestational complications, including preeclampsia or gestational diabetes. BMI was assessed based on the pre-pregnancy weight.

### *2.2 Cell Culture*

Cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in a humidified atmosphere at 37ºC, 5% $CO_2$ and 95% air, in Rosewell Park Memorial Institute (RPMI) 1640 or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (10 IU/ml penicillin, 10 mg/ml streptomycin) in 100 millimeter (mm) culture dishes. Cell stocks were maintained at -80ºC in 90% FBS, 10% dimethyl sulfoxide (DMSO). Trypsin-EDTA was used to lift the cells following a single wash in phosphate buffered solution. The cell lines used: *A)* Human placental choriocarcinoma cell lines which includes BeWo, JAR, JEG-3 (Kohler, Bridson et al. 1971, Pattillo, Gey et al. 1971) express chorionic somatomammotropin, but at much reduced level compared to normal human

term placenta (Nickel and Cattini 1991).

*B)* Human mammary gland adenocarcinoma MCF-7, mammary gland ductal carcinoma T47D, cervical carcinoma HeLa, endometrial adenocarcinoma HEC-1-A and brain glioblastoma U-87 demonstrate evidence of ectopic expression of members of the human CS/GH-V gene family (Wu, Devi et al. 1996, Untergasser, Kranewitter et al. 1997, Khorram, Garthwaite et al. 2001, Raccurt, Lobie et al. 2002, Slater, Cooper et al. 2006, Pandey, Perry et al. 2008, Tuttle, Hugo et al. 2014).

### *2.3 Treatment with DNA Methylation and/or Histone Deacetylation Inhibitors*

For treatment with DNA methylation inhibitor 5-aza-2´ deoxycytidine (azadC) and/or HDAC inhibitor trichostatin-A (TSA), cells were plated at a density of 3 $x10^5$ per well in 35 mm plates, and 24 hours later fed with medium containing 5, 10, 25and 50 µM azadC or 10, 20, 50 and 100 nM TSA for 24 and 18 hours, respectively, before harvesting. For sequential treatment, cells were fed medium with either: (i) 50 µM azadC for 24 hours then refed with medium containing 100 nM TSA for 18 hours; or (ii) 100 nM TSA for 18 hours and then refed with medium containing 50 µM azadC for 24 hours. As controls, cells were treated with a corresponding dose of dimethyl sulfoxide (DMSO) vehicle for comparison in all cases.

### *2.4 RNA Analysis*

Total RNA was extracted using QIAshredder and RNeasy plus Mini kit (Qiagen, Toronto, ON). For reverse transcriptase (RT) polymerase chain reaction (PCR), 1 µg of RNA per reaction was reverse transcribed by the QuantiTect Reverse Transcription kit according to the manufacturer's

instructions (Qiagen). Minus RT reactions were also done to assess genomic DNA contamination. For PCR, 4% HTP or 40% (cell lines) of RT reaction mixture was used with specific primers (Table 1). Detection of a 250 bp product common to all five members of the *h*GH/CS family was assessed using specific primers spanning exon 3 and exon 4 (Table 1) at an annealing temperature of 47ºC for 30 cycles, essentially as described previously (Lytras, Bock et al. 1994). As a result, the presence of unspliced *h*GH/CS transcript or genomic DNA is detected as a 341-343 bp PCR product (Lytras and Cattini 1994). PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.

Identification and relative quantitation of individual *h*CS-A, *h*CS-B and *h*GH-V RNAs was done by real-time RT-PCR using specific primers (Table 1) as described previously (Vakili, Jin et al. 2013). Minus RT reactions were also done using the same primers and thermal cycle conditions as a control for the presence of genomic DNA. Specific amplifications were identified with a single peak in the melting curve and routine verification of a single PCR product visualized by agarose gel electrophoresis. The expression level of each gene was calculated from the standard curve and normalized to either human glyceraldehyde-3-dehydrogenase (GAPDH) or, for azadC and TSA treatments, to β-glucorinidase transcripts.

**Table 1. Primers used for qPCR** (*h* = human)

| RNA | Primer sequence |
|---|---|
| *h*GH/CS | Forward: CAGAAGTATTCATTCCTGCA<br>Reverse: TTTGGATGCCTTCCTCTAG |
| *h*CS-A | Forward: GGCTTCTAGGTGCCCGAGTA<br>Reverse: GCACTGGAGTGGCACCTTCA |
| *h*CS-B | Forward: CAGCAAGTTTGACACAAACTCA<br>Reverse: AGAAGCCACAGCTACCCTCT |
| *h*GH-V | Forward: GTTTGAAAGAAGCCTATATCCTG<br>Reverse: TCACCCTGTTGGAAGGTGTT |
| *h*GAPDH | Forward: TTGATTTTGGAGGGATCTCGC<br>Reverse: GAGTCAACGGATTTGGTCGT |
| *h* β-glucorinidase | Forward: TTGATTTTGGAGGGATCTCGC<br>Reverse: GAGTCAACGGATTTGGTCGT |

### 2.5 *Gene Transfer and Luciferase Assay*

For plasmid DNA transfer, cells were plated ($1.5x10^5$ per 35-mm well) and 24 hours later transfected with hybrid luciferase (Luc) genes (1 µg) using *Trans*IT® - 2020 Transfection reagent according to the manufacturer's protocol (Mirus, Madison, WI). Plasmids containing hybrid *h*CS-A promoter (496 bp) and fire fly luciferase (Luc) reporter genes without (CSp.Luc) or with upstream 263 bp P (263P.CSp.Luc) and downstream 241 bp *h*CS-B enhancer sequences (CSp.Luc.241Enh) were used. The CSp.Luc and 263P.CSp.Luc genes are described elsewhere (Norquay, Jin et al. 2001). The CSp.Luc.241Enh was generated by Ms. Yan

Jin. Briefly, the *h*CS-B 241 bp enhancer fragment was amplified using SV40PCAT-CSBenhancer plasmid DNA as a template (Lytras, Surabhi et al. 1996), using primers containing an AatII restriction enzyme site (CSB-En-Forward: AGCTGCGACGTCGTCTACATTTCAGCTCATCAACTTGG, and CSB-En-Reverse: AGCTCGGACGTCCAGCTGTGAACACATGGGTCTCATCT). The amplicon was digested with AatII and ligated into the AatII site of CSpLuc and downstream of the luciferase gene. The sequence and orientation of the hybrid gene construct was confirmed by sequencing. Cells were co-transfected with 20 ng/well of a hybrid *Renilla* luciferase gene (RLuc) directed by a minimal thymidine kinase promoter (Promega Corp., Madison, WI). Cells were harvested 18 hours post-transfection, and luciferase activity (Luc/RLuc) was measured after cell lysis using the Dual-Luciferase® Reporter (DLR$^{TM}$) Assay System (Promega Corp.) and a photo-counting luminometer.

### *2.6 Protein Immunoblotting*

Nuclear extraction from cell cultures was performed using a Nuclear Extraction Kit according to the manufacturer's instructions (Abcam, #113474). Briefly, after treatment, cells were suspended in PBS (3 milliliter (ml) per 100 mm plate) and centrifuged for 5 minutes at 1000 x g. Cells pellets resuspended in 300 µl of 1x Pre-Extraction Buffer per $10^6$ cells were vortexed vigorously for 10 seconds and centrifuged ( 12000 x g for 1 minute ) Nuclear Extraction Buffer containing DTT and Protease Inhibitor Cocktail -PIC (10 µl per $10^6$ cells) was added to the pellet and incubated on ice for 15 minutes with vortex (5 seconds every 3 minutes). After centrifugation (14000 x g for 10 minutes at 4ºC) the supernatant containing the pure and enriched nuclear fraction was collected. Protein concentrations were determined by a Bradford Protein Assay

(Bio-Rad). For detection of acetyl H3 histones, 5µg (before and after TSA treatment) of nuclear-extracted proteins were analyzed by immunoblotting as described. Proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and immunoblotted with anti-acetyl-H3 antibody (Millipore, #06-599) as well as total histone H3 (CST, #9715) and lamin-B (Abcam, #16048), as controls for change in H3 acetylation following treatment with TSA and loading for nuclear proteins respectively. The detected proteins were visualized using horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) secondary antibody and Enhanced chemiluminescence (ECL) plus immunoblotting detection reagents (Thermo Fischer Scientific Inc.).

### *2.7 Enzyme Linked Immuno Assay (ELISA)*

To determine the global methylation status in placental JEG-3 and non-placental MCF-7 cells following treatment with DNA hypomethylating agent azadC, total DNA was isolated using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON) according to the manufacturer's protocol. The 5- methyl cytosine (mC) content of the total extracted DNA was semi-quantitatively measured using an ELISA kit. The methylated fraction of DNA was detected using the respective capture and detection antibodies using MethylFlash™ Methylated DNA Quantification Kit (Epigentek, #P-1034) according to the manufacturer's protocol and quantified colorimetrically at 450 nm in a micro plate reader. Results are expressed as 5- mC (ng) after azadC treatment relative to independent control (DMSO) values for each group/duration, which are arbitrarily set to 100.

### *2.8 Chromatin Immunoprecipitation (ChIP) Assay*

Assays were processed by Zymo Research Corp. (Irvine, CA). Placental JEG-3 choriocarcinoma cells were cross-linked with formaldehyde at 1% final concentration for 7 minutes at room temperature, and chromatin prepared using the Zymo-Spin ChIP kit (Zymo Research Corp) following manufacturer's instructions. Sonication was performed at the high power setting for 40 cycles (30 seconds on, 30 seconds off) using an Bioruptor Plus (Diagenode Inc., Denville, NJ), yielding a fragment size in the range of 200-700 bp. ChIP assays were performed in triplicate for each sample using 27 µg of chromatin and 5 µg of anti-H3 (Abcam, ab1719) or 5 µg of anti-H3ac (Millipore, 06-599) and anti-H4ac (Millipore 06-598; lot# 2510372). The specificity of the histone antibodies was demonstrated using a normal rabbit immunoglobulin (IgG) polyclonal antibody for immunoprecipitation (Millipore, #PP64B). ChIP DNA was purified using ChIP DNA Clean and Concentrator (Zymo Research), and the relative abundance of HS III, IV and V, downstream *h*CS enhancer elements, P sequences as well as *h*CS-A and *h*GH-V promoter regions in immunoprecipitated DNA and input DNA was quantified by qPCR with sequence-specific primers (Table 2). Values for both hyperacetylated (HAc) histone H3/H4 and IgG were normalized to values for H3, and data for HAc H3-H4 "binding events" are presented relative to IgG control values.

**Table 2. Primers used for ChIP-qPCR**

| RNA | Primer sequence |
|---|---|
| HS V | For: TCCCTCGGACCAGAACAC<br><br>Rev: CCCAGGTAAAAGCAGCATGT |
| HS IV | For: TTCTCAGGGTTTGGGACTGA<br><br>Rev: TGGGAAGAAGTGGTGGACTC |
| HS III | For: CACTGATGAGCTTGGCGTCAC<br><br>Rev: CCTGCCACTTCCGCTCTCCA |
| (CS) $E_{L,A,B}$ | For: TCATCTTTGCGGTCCCTAAC<br><br>Rev: AGCCCTCACTCCCTGAGATT |
| $P_A$ | For: CTGGACTAGCACCCCAGACTCATCAT<br><br>Rev: TCGATGACACCCCTCTTGGATCCTCC |
| CS-Ap | For: GAGGAGCTTCTAAATTATCCAC<br><br>Rev: CAGTTCTCTCTCCCTGCTTGA |
| $P_V$ | For: TTCCTGAAACATTATCGGACC<br><br>Rev: TTGGATCCTCACACAGGCGG |
| GH-Vp | For: AGTGGCCCCAGGCCTAAACA<br><br>Rev: CCTTCTCTCTCGCTGCTTCT |

### *2.9 Statistical Analysis*

All studies were done in triplicate unless stated otherwise, with at least two determinations per sample. Statistical analysis was done using Prism® software. For two category comparisons

unpaired t-tests were used, and for multiple comparisons one-way analysis of variance (ANOVA) was used with a post-hoc Tukey-Kramer test as appropriate. A value of $p<0.05$ is considered statistically significant and is represented in Figures as: * or #, $p<0.05$; ** or ##, $p<0.01$; *** or ###, $p<0.001$.

# CHAPTER 3

## 3.1 *Detection and comparison of hCS-A, hCS-B and hGH-V RNA levels in tumor cells of placental and non-placental origin versus human normal term placenta*

Pregnancy hormones including CS and GH-V are expressed in the syncytiotrophoblast layer of the human placenta (McWilliams and Boime 1980). The rise in circulating *h*CS and *h*GH-V protein concentrations in maternal serum during gestation (Frankenne, Closset et al. 1988) reflects placental growth (Braunstein, Rasor et al. 1980) and/or enhanced *"transcriptional activation rather than amplification"* of the *h*CS/GH-V genes (Boime, McWilliams et al. 1976, McWilliams, Callahan et al. 1977). Studies have demonstrated that transcripts from *h*CS-A and *h*CS-B genes (both independently encoding mature CS) is initiated with differentiation of placental mono-nucleated cytotrophoblasts to multi-nucleated syncytiotrophoblasts (Klassen, Nachtigal et al. 1989) and GH-V which encodes placental growth hormone directly sub localize to the syncytiotrophoblastic epithelium of the human term placenta (Hoshina, Boothby et al. 1982, Cooke, Ray et al. 1988, Liebhaber, Urbanek et al. 1989, Scippo, Frankenne et al. 1993).

As previously stated in Section *1.6.1, "There is a developmentally regulated switch in the relative expression"* of *h*CS-A and *h*CS-B transcripts during pregnancy; starting at comparable levels at 8 weeks of pregnancy and both encoding identical CS protein, *h*CS-A transcripts are 5-

fold more abundant than those of *h*CS-B by term in uncomplicated pregnancies (MacLeod, Lee et al. 1992), and *h*GH-V transcripts levels are several orders of magnitude lower than *h*CS-A and *h*CS-B transcripts (Chen, Liao et al. 1989). CS and GH-V serve as diagnostic markers for normal progression of pregnancy (Handwerger 1991). However, this pattern of *h*CS/GH-V expression is not always conserved in normal placental tissues or cells based on evidence which suggests that level of *h*CS/GH-V transcripts are significantly decreased in pregnancies complicated by maternal obesity (Vakili, Jin et al. 2013) or altered in pregnancies associated with "small- and large- for gestational age" infants (Mannik, Vaas et al. 2010). Furthermore, an altered expression pattern is also observed in abnormal placental tissues or cells, including hydatidiform mole and choriocarcinoma cell lines (Szulman and Surti 1978, Hoshina, Hussa et al. 1984, Hoshina, Boothby et al. 1985, Morrish, Honore et al. 1992, Lytras, Bock et al. 1994). Human placental tumor cell lines consist of "*mitotically active cytotrophoblasts with moderate degrees of differentiation"* (Ringler and Strauss 1990), endogenously express *h*CS/GH-V transcripts at relatively low levels compared to normal term placenta (Nickel and Cattini 1991, Nachtigal, Bock et al. 1992, Lytras, Bock et al. 1994), and offers a good model for studying *h*CS/GH-V gene regulation. Studies have also demonstrated ectopic *h*CS/GH-V gene expression in human endometrial tissue (Slater, Cooper et al. 2006), normal human mammary gland (Mol, Henzen-Logmans et al. 1995, Raccurt, Lobie et al. 2002), normal and neoplastic human lymphoid tissues and cells (Hattori, Shimatsu et al. 1990, Wu, Devi et al. 1996), human ovaries (Schwarzler, Untergasser et al. 1997, Silva, Figueiredo et al. 2009), human testis (Untergasser, Kranewitter et al. 1997) and also in non-placental endometrial carcinoma cell lines (Khorram, Garthwaite et al. 2001, Sbracia, Scarpellini et al. 2004, Pandey, Perry et al. 2008) and adenocarcinoma cells such

as the breast cancer MCF-7  cell line (Tuttle, Hugo et al. 2014).

*The expression profile of individual hCS-A, hCS-B and hGH-V transcripts using specific primers have not been reported in placental and non-placental tumor cells.*

## 3.2 RESULTS

### 3.2.1 *Detection of hCS-A, hCS-B and hGH-V transcripts in placental and non-placental tumor cells*

Total *h*CS/GH-V RNA was isolated from normal human term placenta (HTP) as well as human placental (BeWo, JAR and JEG-3) and non-placental cervical carcinoma HeLa, breast adenocarcinoma MCF-7 and ductal carcinoma T-47D, uterine adenocarcinoma Hec1A and glioblastoma U-87 cells. Transcripts common to *h*GH/CS genes and combining exon 3 and 4 sequences, as well as human GAPDH RNA were assessed using specific primers (Table 1) by reverse transcription polymerase chain reaction (RT-PCR) and gel electrophoresis. Relative to HTP, the presence of low levels of *h*GH/CS transcripts was confirmed in examples of both human placental and non-placental tumor cells. The exception was U-87 glioma cells where *h*GH/CS transcripts were not detected under equivalent conditions (Figure 3.2 A).

Based on the expression levels of the total *h*CS/GH-V transcripts; Individual *h*CS-A, *h*CS-B and *h*GH-V gene expression was investigated in placental (BeWo, JAR, JEG-3) and non-placental HeLa, MCF-7, U-87 tumor cells. This reflects a selection of available placental tumor cells and a range of expression levels in non-placental cell lines. This includes JEG-3 and MCF-7 that

appear to have comparable levels of *h*CS/GH-V RNA. RNA was assessed by qPCR using specific primers for *h*CS-A, *h*CS-B and *h*GH-V transcripts (Table 1), and products were routinely examined by gel electrophoresis; a representative image is shown in (Figure 3.2 B). For quantitation, the values for *h*CS-A, *h*CS-B and *h*GH-V transcripts were normalized to human GAPDH RNA levels. Results are presented relative to levels in JEG-3, which are arbitrarily set to 1.0 (Figure 3.2 C). Expression of *h*CS-A, *h*CS-B and *h*GH-V genes was detected in all cell lines, but this was a fraction of that observed in HTP based on a comparison with levels in JEG-3 cells. Relative levels of individual *h*CS-A, *h*CS-B and *h*GH-V transcripts were all significantly greater in BeWo and JAR *versus* JEG-3 cells. However, a more variable pattern of relative expression was seen with JEG-3 compared to non-placental tumor cells; while *h*CS-A RNA levels were greater in HeLa and MCF-7 cells, *h*CS-B transcripts were greater only in HeLa cells and *h*GH-V transcripts were greater in MCF-7 cells alone (Figure 3.2 C). There was also no significant difference in individual *h*CS-A, *h*CS-B and *h*GH-V RNA levels between JEG-3 and U-87 cells.

***Figure 3.2 A***

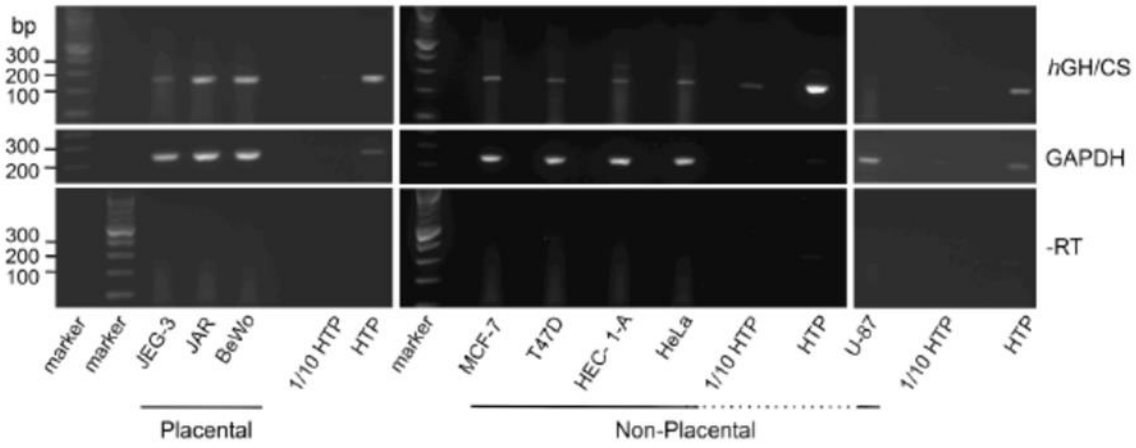



***Figure 3.2 B***

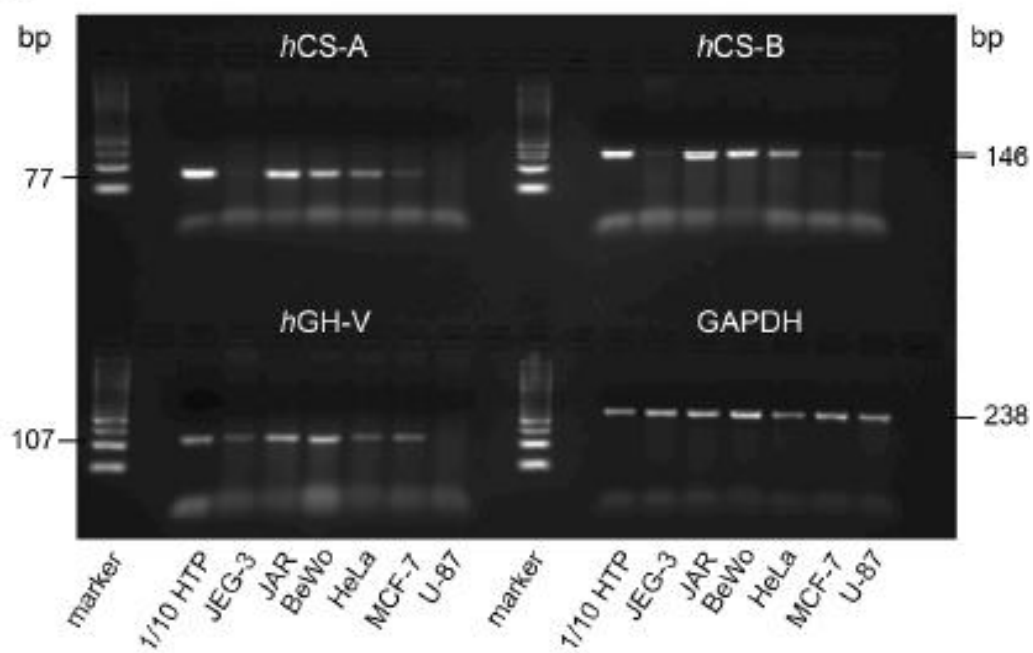



***Figure 3.2 (A)*** Assessment of total human (*h*) GH/CS RNA and relative levels of *h*CS-A, *h*CS-B

and *h*GH-V transcripts in placental and non-placental cells. RNA was isolated from tumor cells indicated as well as human term placenta (HTP) samples, and was assessed by RT-PCR using primers common to the five human (*h*) GH/CS genes that span exon 3 and 4 sequences to yield a 250 bp amplicon (or 341-343 bp unspliced RNA or genomic DNA product) as well as GAPDH. Minus RT reactions were also assessed. All products as well as 100 bp markers were visualized by agarose gel electrophoresis and ethidium bromide staining as shown in the above representative image. ***(B)*** RNA was isolated from placental and non-placental tumor cells and assessed by qPCR using specific primers to *h*CS-A, *h*CS-B and *h*GH-V as well as GAPDH transcripts. All products as well as 50 bp markers were visualized by agarose gel electrophoresis and ethidium bromide staining as shown in this representative image.

***Figure 3.2 C***

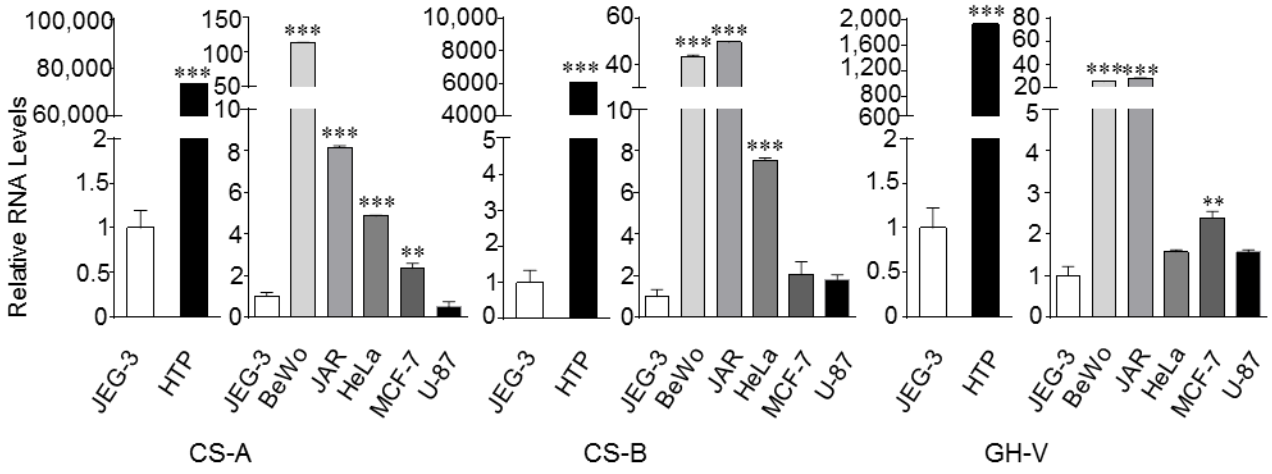



***Figure 3.2 C:***

Human CS-A, CS-B and GH-V as well as GAPDH transcripts were assessed as in Figure 3.1B, and the latter was used to normalize the data. Results are expressed as fold-difference in mean values relative to JEG-3 RNA levels, which has been arbitrarily set to 1.0, and error bars indicate standard error of the mean (n=3). Comparisons of RNA levels between JEG-3 and HTP was assessed by one-way ANOVA with post Tukey-Kramer Multiple Comparisons test (**, $p<0.01$, ***, $p<0.00$

# CHAPTER 4

## 4.1 *Comparison of hCS promoter, P sequence and 3´ -Enhancer activity in transfected placental and non-placental tumor cells as an indication of transcription factor availability*

Appropriate expression of *h*CS/GH-V genes from GH/CS locus have been linked in parts to proximal *cis* acting regulatory elements and associated *trans*-acting factors which includes:

**(i)** Local repeat elements, termed **P** sequences, located approximately 2 kilobases (kb) upstream of the placental *h*CS-L, *h*CS-A, *h*GH-V, *h*CS-B promoters but not pituitary *h*GH-N promoter (Chen, Liao et al. 1989). The 263-bp core element (263P) of **P** sequences was shown to blunt the *in vitro* activity of the CS-A promoter in pituitary (GC) but not placental (JEG-3) cells after gene transfer (Nachtigal, Nickel et al. 1993). Cell culture-based studies have suggested that the 263P region and its corresponding factors represses CS-A promoter in pituitary GC tumor cells but not in placental JEG-3 cells (Nachtigal, Nickel et al. 1993, Norquay, Jin et al. 2001, Norquay, Yang et al. 2003, Norquay, Yang et al. 2006). *In vivo* studies have also suggested a stimulatory role for the P element in *h*CS activation. This was demonstrated by HSI,II/P/hGH/P transgenic lines expressing pituitary specific *h*GH mRNA in the mouse placenta (Elefant, Su et al. 2000). Thus, *in vivo* and *in vitro* data suggest that 263P element may have functional significance, however, the direction of response and how this is affected by tissue location is yet to be determined for individual members of the *h*GH/CS gene family.

**(ii)** A transcriptional enhancer was identified in a 1022 bp region that lies ~2 kb downstream of the *h*CS-B gene (Rogers, Sobnosky et al. 1986). Fragments of the 1022 bp region were tested for enhancer activation of a heterologous simian virus 40 (SV40) promoter in JEG-3 cells. Fragments spanning 1-210 and 103-241 bp retained 94% and 93% of the enhancing activity of the full length 1022 bp sequence respectively (Walker, Fitzpatrick et al. 1990). Enhancer activity was localized to 241 bp nucleotides (nts) 1-241, of the 1022 bp region which has been shown to enhance homologous or heterologous promoter activity by 300- to 400-fold in placental trophoblast cells *in vitro* (Walker, Fitzpatrick et al. 1990, Jacquemin, Oury et al. 1994, Lytras and Cattini 1994). The 241 bp *h*CS-A "enhancer" region was not equally efficient *in vitro* (Rogers, Sobnosky et al. 1986, Jacquemin, Alsat et al. 1996, Lytras, Surabhi et al. 1996). The *h*CS 'enhancer' regions contain hyperacetylated H3-H4 histones in primary placental and choriocarcinoma (BeWo) cells (Kimura, Sizova et al. 2007). Detailed analysis of the 241 bp of *h*CS-B 3´-Enh region suggest involvement of *trans*-acting factors necessary for activated *h*CS expression (Walker, Fitzpatrick et al. 1990, Jacquemin, Oury et al. 1994, Jiang and Eberhardt 1995, Jiang, Shepard et al. 1995, Lytras, Detillieux et al. 2011, Vakili, Jin et al. 2013). In addition to the local regulatory elements, basal *h*CS promoter activity was demonstrated in placental JEG-3 cells between nucleotides -142 and -129 which bind ubiquitous Sp1 protein (Fitzpatrick, Walker et al. 1990). There is evidence to support the presence of putative AP2 binding sites within the first 325 bp of the CS-A promoter (Richardson, Langland et al. 2000). Mutations in AP2 binding sites resulted in 60-80% decrease in *h*CS promoter activity compared to the wild type promoter activity (Richardson, Langland et al. 2000).

Compared to the normal HTP *h*CS-A, *h*CS-B and *h*GH-V genes are expressed at an extremely

low level in placental JEG-3 cells (Figure 3.2C) (Lytras, Bock et al. 1994). Interestingly, low but comparable levels of *hCS/GH-V* RNA was detected in JEG-3 cells and in non-placental MCF-7 and U87 cells. This suggests that the *placental origin* of the JEG-3 is not apparent in the response and low levels of expression.

## 4.2 RESULTS

### 4.2.1 *The downstream CS enhancer region shows preferential activity in placental versus non-placental tumor cells*

Expression of *h*CS have been linked to local *cis* DNA regions including upstream P sequences and downstream enhancer (3´-Enh) regions and their associated *trans*-acting factors (Vakili, Jin et al. 2013). The activity of local regulatory regions were determined in placental (JEG-3, BeWo) and non-placental (MCF-7 and U-87) cells by gene transfer assays. Cells were transiently transfected using: **1)** 492/+6 of CS-A gene immediate 5´-flanking region (CS-Ap) upstream of the firefly Luc gene (CSp.Luc); **2)** A hybrid firefly luciferase gene directed by the 263P fragment upstream of the 492/+6 of CS-A gene (263P.CSp.Luc); and **3)** 241-Enh element downstream of the hybrid firefly luciferase gene directed by 492/+6 of CS-A gene (CSp.Luc.241Enh). To control for DNA uptake, cells were co-transfected with *Renilla* luciferase gene (pRLTKp.Luc) and luciferase activity was measured after 18 hours (Figure 4.2). Corrected (Luc/RLuc) values are expressed relative to CSp.Luc activity, which is arbitrarily set to 1.

In placental (JEG-3) and non-placental (MCF-7 and U-87) cell lines, the presence of 263P had a modest (<2-fold) but significant increase in CS-A promoter activity. However, 263P repressed CS-A promoter activity (<2-fold) in placental BeWo cells. The repressive *in vitro* activity of 263P observed in BeWo cells does not reflect the enhancer activity of P sequence to stimulate expression of the *h*GH-N gene in placenta of transgenic mouse carrying HSI,II/P/hGH/P transgene (Elefant, Su et al. 2000). The presence of the 241-Enh element resulted in significant CS-A promoter activity in placental but not non-placental tumor cells. A significant and more robust 5 to 12-fold increase in activity was observed in the presence of 3´-Enh sequences in placental JEG-3 and BeWo cells. A modest stimulation (1.3 fold) was seen in non-placental MCF-7 but not U-87 cells. Thus, consistent 3´-Enh activity was seen in the two placental but not non-placental cell lines tested.

***These data raise the possibility that the placental enhancer factors are present in BeWo and JEG-3 cells but unlike in the transfected luciferase reporter gene the cis-elements in the endogenous hCS genes are not accessible in the context of chromatin.***

***Figure 4.2***

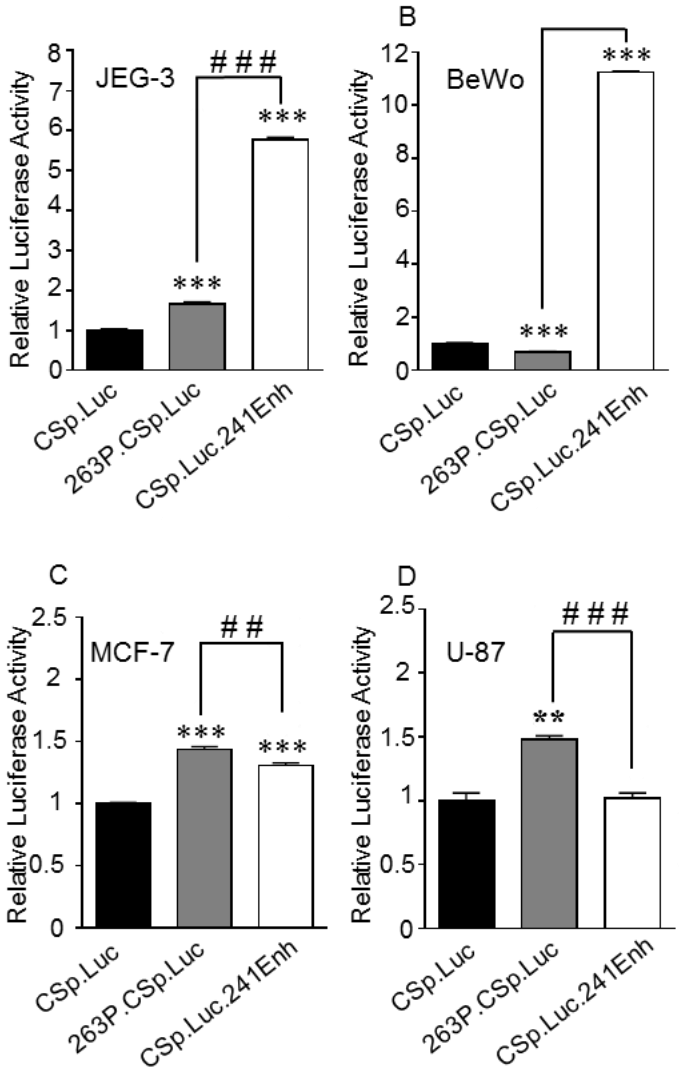



***Figure 4.2:***

The downstream enhancer region shows preferential activity in transfected placental versus non-placental tumor cells. Hybrid *h*CS-A promoter (CSp)/luciferase (Luc) genes without (CSp.Luc) or with the 263 bp P sequence element (263P.CSp.Luc) or 241 bp *h*CS-B downstream enhancer element (CSp.Luc.241Enh) were used to transiently transfect placental (JEG-3, BeWo) and non-placental (MCF-7, U-87) cells. To control for DNA uptake cells were co-transfected with a hybrid Renilla luciferase gene (RLuc). Results are expressed as mean values (Luc/RLuc) relative to CSp.Luc activity, which was arbitrarily set to 1, and error bars represent standard error of the mean (n=6). The mean values for CSp.Luc in JEG-3, BeWo, MCF-7 and U-87 were 1.895 ±

0.066, 0.788 ± 0.039, 3.110 ± 0.033 and 2.356 ± 0.148, respectively. Results for promoter activity were analyzed by one-way ANOVA with post Tukey-Kramer Multiple Comparisons test (**, $p<0.01$, ***, $p<0.001$).

# CHAPTER 5

## 5.1 *Effect of DNA methylation and/or histone deacetylation inhibition on hCS/GH-V gene expression in placental and non-placental cells, as a means to increase transcription factor accessibility*

Eukaryotic gene expression is tightly controlled by DNA methylation and/or histone modifications (Cedar and Bergman 2009). DNA methylation and histone modifications have garnered much interest as possible mechanisms underlying tissue-specific gene expression (Bird 2002, Shiota 2004, Yagi, Hirabayashi et al. 2008). For example, examination of DNA methylation and histone H3 acetylation profiles provide clear evidence that liver-specific expression of genes associated with DNA and histone modifications (Imai, Kikuchi et al. 2009).

DNA methylation is associated with repression of transcription (Cedar 1988), and for the ability to interfere with transcription factor binding (Ehrlich and Ehrlich 1993). In some instances, DNA methylation and associated methyl-CpG binding proteins at the methylated sites can mediate histone modifications by recruiting HDACs to methylated DNA (Nan, Ng et al. 1998, Ng, Zhang et al. 1999, Wade, Gegonne et al. 1999).

Assay of the *h*CS and *h*GH-V genes showed similar patterns DNase I digestion patterns but increased sensitivity in normal human term placenta *versus* choriocarcinoma JEG-3 and BeWo cells; increased sensitivity was interpreted as evidence of a more open chromatin structure (Nickel and Cattini 1996). Increased levels of nuclease were required to see the same digestion

pattern in human non-placental cervical carcinoma HeLa cell nuclei/chromatin, suggesting a more condensed chromatin structure containing the *h*CS/GH-V gene locus (Nickel and Cattini 1996). Together, these observations suggest that an *"open"* chromatin structure correlates with transcriptional activity and there is a possibility that undefined mechanisms such as DNA methylation and histone modifications are responsible for differences in CS and GH-V gene expression by human term placenta *versus* choriocarcinoma cells (Nickel and Cattini 1996, Cattini, Yang et al. 2006).

Studies using primary human trophoblast and choriocarcinoma cells have indicated that the *h*GH/CS locus is a target for DNA and histone modifications. Activation of the placental members of the *h*GH/CS gene family reflect distinct patterns of histone H3-H4 hyperacetylation at the placental LCR and local regulatory regions, which possess enhancer activity (Elefant, Su et al. 2000, Jin, Norquay et al. 2004, Kimura, Liebhaber et al. 2004, Kimura, Sizova et al. 2007). An assessment of DNA methylation status in the *h*GH/CS gene locus using methylation sensitive restriction enzymes suggested that undermethylation may play a role in the *h*GH/CS gene expression in placenta *versus* non-placental leukocytes (Hjelle, Phillips et al. 1982). This study was limited by the fact that it was not able to distinguish the methylation pattern in the distal and proximal regulatory elements. Thus, the approach used was unable to detect any differences between hydatidiform mole DNA compared to term placenta even though moles make little or no CS (Hjelle, Phillips et al. 1982).

To investigate the relevance of DNA methylation and histone acetylation, placental and non-placental tumor cells were treated with a hypomethylating agent (azadC) and deacetylating agent (TSA). AzadC has been reported to reactivate gene expression but is cytotoxic at higher doses

(Jabbour, Issa et al. 2008). Studies have demonstrated usage of 1, 5 and 10 µM azadC in JAR and MCF-7 cells for 3-7 days (Dom, Galdo et al. 2013). As discussed in Section ***1.5*** of this thesis, the bimodal pattern of DNA methylation is maintained following DNA replication during the cell cycle. The doubling time in choriocarcinoma cells is approximately 24-28 hours (Ringler and Strauss 1990). Thus, we selected doses ranging from 5 to 50 µM azadC for a period of 24 hours. The deacetylating agent TSA has been used in studies to assess the effects of histone acetylation on gene expression (Zhang, Fatima et al. 2005, Dom, Galdo et al. 2013). Histone hyperacetylation has been linked to increased gene expression (Bowman and Poirier 2015). TSA is known for inhibiting HDAC activity, inducing cell cycle arrest and cytotoxicity in human neuroblastoma cell lines (Furchert, Lanvers-Kaminsky et al. 2007). The dose of TSA used was based largely on previous studies. For example, 100 nM TSA for 48, 72 hours was used in MCF-7 cells and up to 1.65 µM TSA for 12 hours has been used in choriocarcinoma JAR cells (Zhang, Fatima et al. 2005, Dom, Galdo et al. 2013). Thus, dose range of 10, 20, 50 and 100 nM TSA for 18 hours was selected for this study. A shorter duration was selected to offset potential cytotoxic effects of TSA and thereby assist with interpretation of any data. In section ***1.6*** of this thesis, the interplay between DNA methylation and histone acetylation is discussed. Both TSA and azadC have been used to derepress the LHR gene expression (Zhang, Fatima et al. 2005). In this study sequential treatment with azadC followed by TSA was also pursued based on evidence indicating that DNA methylation recruits HDACs thereby contributing to repression of gene expression (Wolffe 1998, Nguyen, Gonzales et al. 2001).

## 5.2 RESULTS

### 5.2.1 *Differential effects of DNA demethylase and HDAC inhibition on hCS-A, hCS-B and hGH-V RNA levels*

To increase accessibility of placental enhancer transcription factors to the *cis* regulatory elements in the *h*CS/GH gene locus placental JEG-3 cells were treated with increasing concentrations of either azadC (5, 10, 25 and 50 μM) for 24 hours or TSA (10, 20, 50 and 100 nM) for 18 hours. Levels of *h*CS-A, *h*CS-B and *h*GH-V RNA were then assessed by qPCR (Figure 5.2). For controls, cells were treated with DMSO vehicle under identical conditions for each treatment group, and RNA levels were normalized relative to β-glucorinidase transcripts. Treatment with 25 μM azadC or greater resulted in modest but significant and consistent increases in *h*CS-B and *h*GH-V but not *h*CS-A RNA levels in JEG-3 cells (Figure 5.2 A). Human GH-V transcripts showed greater sensitivity to TSA than *h*CS-A and B, as levels were increased ~2-fold with 10 and 20 nM TSA treatment. However, *h*GH-V as well as *h*CS-A and *h*CS-B levels were all significantly increased ~5-fold with 50 nM TSA, and a further increase (equivalent to 7 to 15-fold) was seen with 100 nM TSA treatment (Figure 5.2 B).

***Figure 5.2 A***

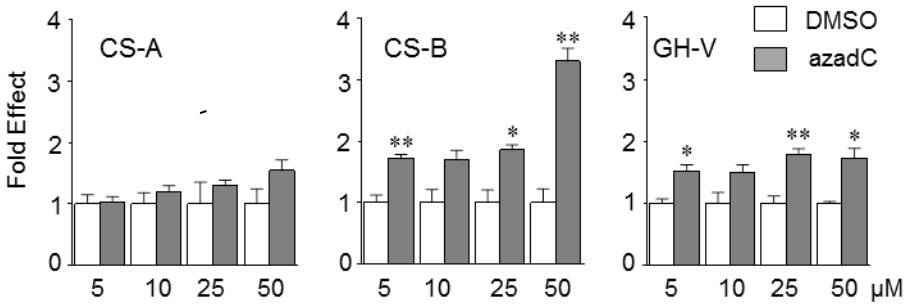



***Figure 5.2 B***

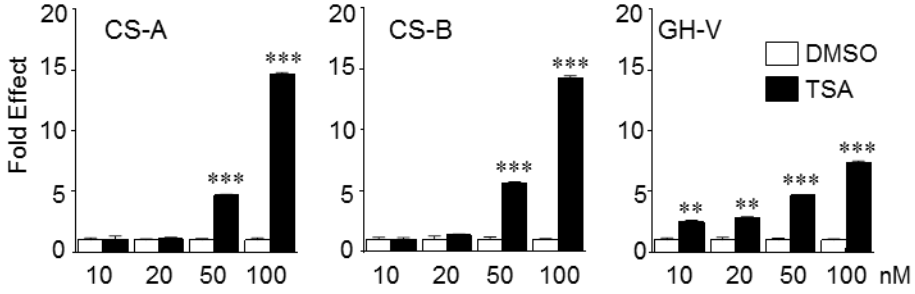



***Figure 5.2 A and 5.2 B:***

Differential effects of DNA demethylase and HDAC inhibition on *h*CS-A, *h*CS-B and *h*GH-V RNA levels. Placental JEG-3 cells were treated with increasing concentrations of the **A)** hypomethylating agent 5-aza-2´ deoxycytidine (azadC) or vehicle DMSO for 24 hours, or **B)** HDAC inhibitor Trichostatin-A (TSA) or DMSO for 18 hours. RNA was assessed by qPCR using specific primers for *h*CS-A, *h*CS-B and *h*GH-V as well as glucorinidase transcripts, which was used to normalize the data. In all cases, mean values and standard error of the mean were determined, and results are expressed as fold-effect of azadC or TSA treatments relative to independent control (DMSO) values, which are arbitrarily set to 1.0. Data were analyzed by t-test, and significant differences between DMSO control and azadC or TSA treatment groups are indicated by **, $p<0.01$ and ***, $p<0.001$ (n=6).

### *5.2.2 Levels of hCS/GH-V transcripts are increased synergistically with sequential azadC and TSA treatments in placental JEG-3 but not non-placental MCF-7 cells*

The effect of sequential 50 µM azadC (24 hours) and 100 nM TSA (18 hours) on *h*CS/GH-V transcripts in JEG-3 and MCF-7 cells was assessed by qPCR, given the individual effects of DNA demethylase and HDAC inhibition in JEG-3 cells. Synergistic and significant increases in *h*CS-A (~37-fold), *h*CS-B (~44-fold) and *h*GH-V (~15-fold) RNA levels were observed in JEG-3 cells treated with azadC and TSA versus TSA alone (Figure 5.2 C). By contrast, more modest and additive ~3-fold increases in *h*CS-A and *h*CS-B were seen in MCF-7 cells (Figure 5.2 D), while *h*GH-V RNA levels were not increased but rather decreased by ~50% (Figure 5.2 D).

To assess the specificity of the response in placental JEG-3 cells, the sequential study was repeated with the reversal of the sequential treatment regimen (100 nM TSA for 18 hours then 50 µM azadC for 24 hours). By contrast to the effect of pretreatment with azadC, post-treatment with azadC mutes the stimulatory effects of TSA on *h*CS/GH-V transcripts (Figure 5.2 E).

These studies indicate that the DNA methylation and/or histone acetylation status of the *h*CS/GH-V gene chromatin structure contributes to low levels of expression in choriocarcinoma cells. Presumably, the chromatin is more condensed and less accessible to transcription factors.

***Figure 5.2 C***

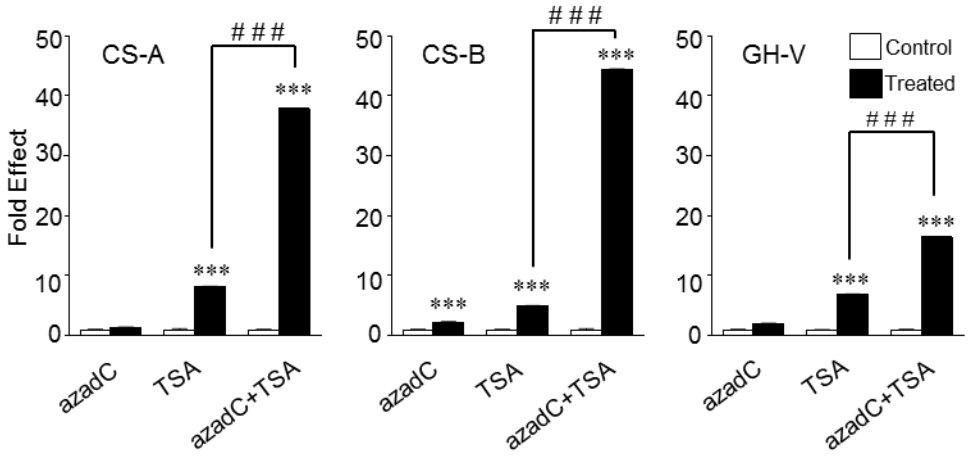



***Figure5.2D***

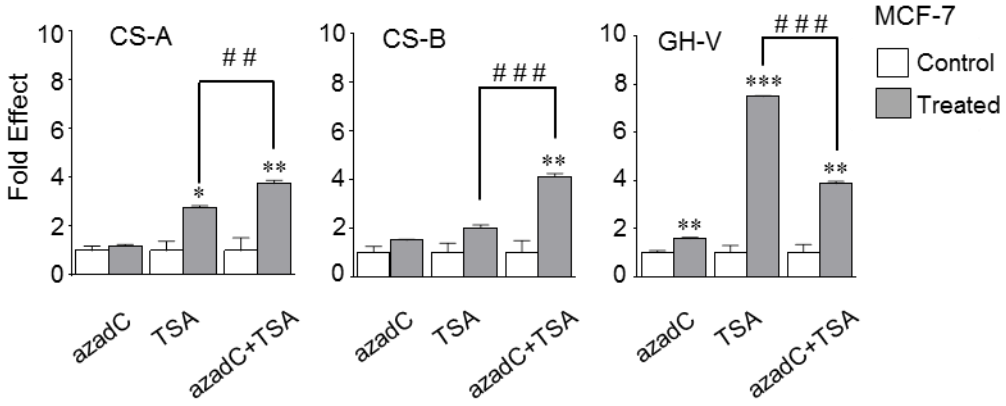


***Figure 5.2 E***

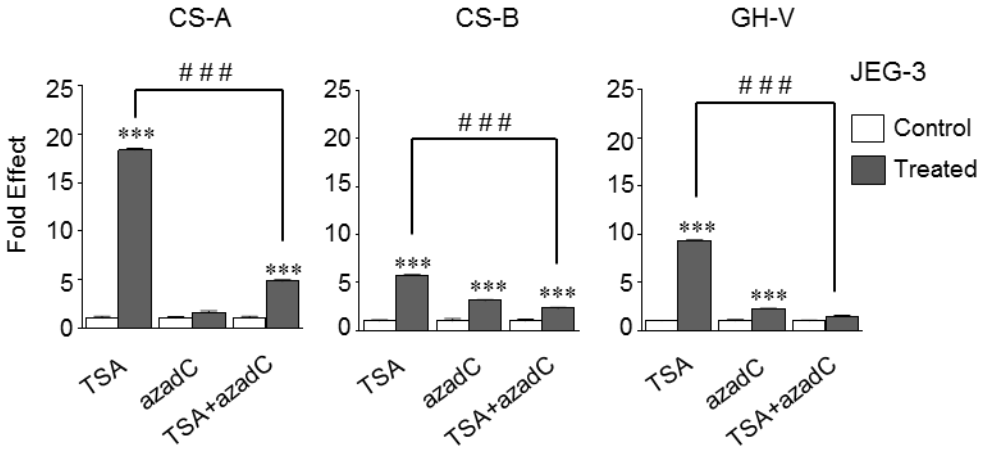



***Figure 5.2***

Human CS-A, CS-B and GH-V RNA levels are increased specifically in placental JEG-3 cells with sequential azadC and TSA treatments. **(C)** JEG-3 cells and **(D)** non-placental MCF-7 cells were treated with: 50 µM 5-aza-2´ deoxycytidine (azadC) for 24 hours; 100 nM trichostatin-A (TSA) for 18 hours; or azadC for 24 hours followed by TSA for 18 hours (azadC+TSA). **(E)** JEG-3 cells were also treated with TSA for 18 hours followed by azadC for 24 hours (TSA+azadC). In all studies **(C-E)**, cells were treated with vehicle (DMSO) for the corresponding length of time used for azadC and/or TSA (Control). Levels of *h*CS-A, CS-B and GH-V RNA were assessed by qPCR as described in Figure 4. Mean values and standard error of the mean were determined, and results are expressed as fold-effect of azadC, TSA or azadC/TSA treatments relative to independent control values for each treatment group/duration, which are arbitrarily set to 1 (n=4-6). Data were analyzed by t-test. Significant differences between control and azadC or TSA treatment groups are indicated by **, $p < 0.01$ and ***, $p < 0.001$. Significance between TSA and azadC/TSA treatment groups are indicated by ###, $p < 0.001$.

### *5.2.3 Decrease in DNA methylation level after treatment with hypomethylating agent azadC and increase in histone H3 hyperacetylation status following treatment with inhibitor of histone deacetylase TSA*

After treatment with 50 µM hypomethylating azadC, global DNA methylation in placental JEG-3 and non-placental MCF-7 cells were analyzed by ELISA assay. For controls, cells were treated with DMSO vehicle under identical conditions for 24 hours. The quantification of the amount of 5- methyl cytosine (mC) showed a significant decrease in JEG-3 cells. However, although there was an apparent decrease observed in MCF-7 cells it was not significant.

***Figure 5.2 F***

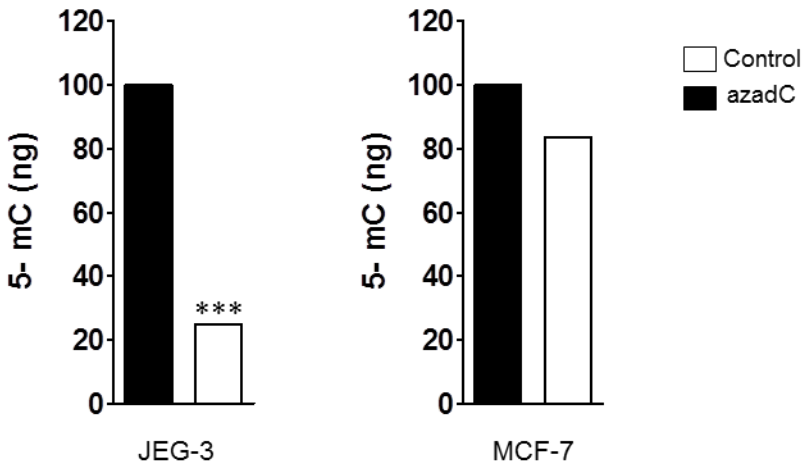



***Figure 5.2 F***

Placental JEG-3 and non-placental MCF-7 cells were treated with 50 µM of DNA hypomethylating agent azadC. The 5- mC content of the total extracted DNA was semi-quantitatively measured by a DNA quantification kit. The methylated fraction of DNA was

detected using the respective capture and detection antibodies and quantified colorimetrically at 450 nm in a micro plate reader. Mean values and standard error of the mean were determined, and results are expressed as 5- mC (ng) after azadC treatment relative to independent control values for each group/duration, which are arbitrarily set to 100 (n=4). Data were analyzed by t-test. Significant differences between control and azadC treatment groups are indicated by ***, p <0.001.

Protein immunoblotting was done to assess overall effects of TSA on histone H3 acetylation status. JEG-3 and MCF-7 cells were treated with DMSO and 100 nM of histone deacetylase inhibitor TSA for 18 hours. Cells were harvested and nuclear proteins were extracted. An increase in overall H3 acetylation was observed in TSA treated placental and non-placental cells by protein immunoblotting using specific antibodies to anti-acetyl-H3 relative to total H3.

***Figure 5.2 G***

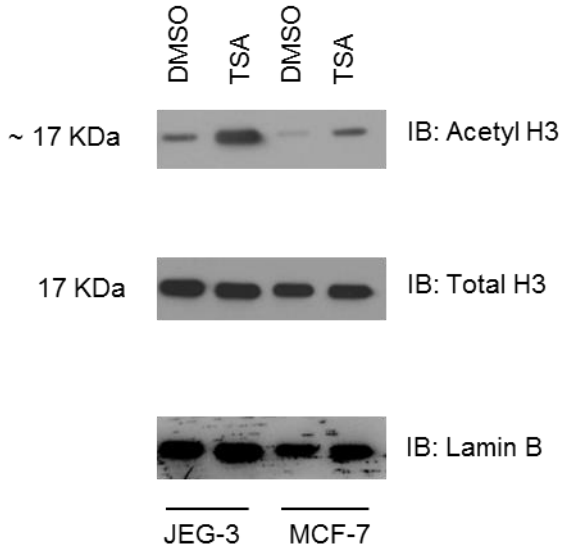



***Figure 5.2 G***

Nuclear-extracted proteins (5µg) were separated by 15% sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membranes, and immunoblotted with Anti-Acetyl-H3 antibody (Millipore, #06-599), total histone H3 (CST, #9715) and Lamin-B (Abcam, #16048). The detected proteins were visualized using horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) secondary antibody and ECL plus immunoblotting detection reagents (Thermo Fischer Scientific Inc., Nepean, ON, Canada). This was done in duplicate. Figure above is representative pattern of each treatment.

### *5.2.4 Significant increase in H3/H4 hyperacetylation of the hCS$_{A, B, L}$ 3´ Enh sequences in placental JEG-3 cells after sequential azadC and TSA treatment*

As mentioned in Section *5.2.2*, *h*CS-A, *h*CS-B and *h*GH-V RNA levels are synergistically increased in JEG-3 cells after sequential azadC and TSA treatment. The response was specific as when the study was repeated with the reversal of the sequential treatment regimen, it failed to elicit a similar response. As already discussed in Section *5.1*, histone H3-H4 hyperacetylation at local and more remote (LCR) regulatory regions have been linked to placental gene activation. Based on the preferential response in Section *5.2.2*, histone H3-H4 hyperacetylation status was assessed in JEG-3 cell chromatin *in situ* by ChIP assay (Figure 7). Primers were selected to assess the *h*CS/GH-V P and promoter sequences as well as HS III, IV and V from the *h*GH/CS LCR, and combined *h*CS $_{A, B, L}$ 3´ -Enh regions (Table 2). There was an increase in histone H3-H4 hyperacetylation of the *h*GH/CS gene locus with TSA as well as sequential azadC and TSA treatments relative to IgG control values. Based on the increase in hCS/GH-V RNA levels (Figure 5.2C), this suggests that increased hyperacetylation makes the *h*GH/CS locus chromatin more open and accessible for transcription-related activity. Significant increases in H3-H4 hyperacetylation with sequential treatment was observed in the *h*CS $_{A, B, L}$ 3´ -Enh sequences, the *h*CS-A and *h*GH-V promoters, P sequences of *h*GH-V, HS III and IV but not HS V of the

*h*GH/CS LCR (Figure 5.3).

***Figure 5.3***

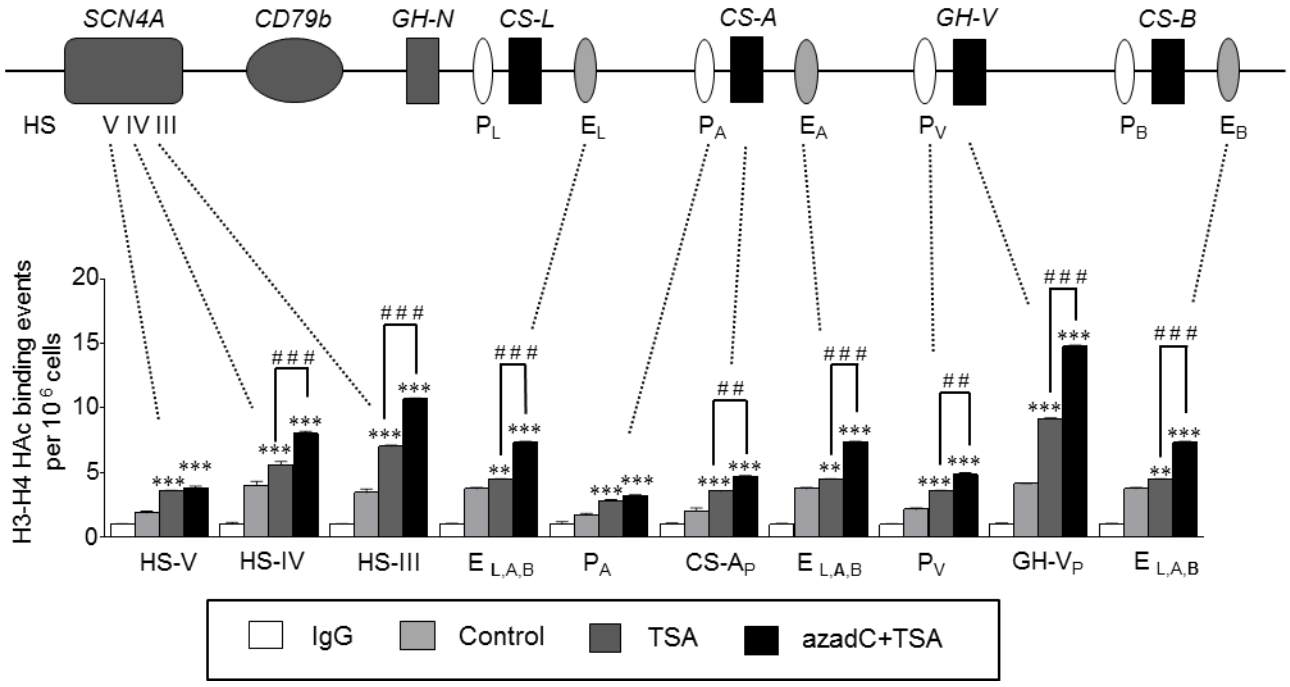



***Figure 5.3:***

Histone H3-H4 hyperacetylation increases across the *h*GH/CS locus *in situ* with sequential azadC/TSA treatment of JEG-3 cells. ChIP assay was performed with antibodies specific to hyperacetylated (HAc) H3 and H4 on chromatin isolated from JEG-3 cells treated without (control) or with 100 nM trichostatin-A (TSA) for 18 hours, or 50 µM 5-aza-2´ deoxycytidine (azadC) for 24 hours then TSA for 18 hours (azadC+TSA). Quantitative PCR was performed on both input and immunoprecipitated (bound) chromatin fractions (n=3) with primer sets designed to detect regions of the *h*GH/CS LCR (HS III, IV and V), downstream enhancer regions common to *h*CS-L, CS-A and CS-B ($E_{L, A, B}$), P sequences upstream of *h*CS-A ($P_A$) and h GH-V (PV) and the proximal promoter regions for *h*CS-A (CS-Ap) and *h*GH-V (GH-Vp).Results are presented as HAc H3-H4 binding events relative to IgG control values for each primer set, which is arbitrarily set to 1. Values for HAc H3-H4 and IgG were all normalized to values for H3. Statistical analysis were performed compared to the control value for each primer set by one-way

ANOVA with the post Tukey-Kramer Multiple Comparisons test (**, $p < 0.01$ and ***, $p < 0.001$). Comparison of TSA and azadC + TSA for each primer pair was assessed by t-test; ###, $p < 0.001$.

# CHAPTER 6

# DISCUSSION

## 6.1 *Expression of hCS-A, hCS-B and hGH-V transcripts in normal term placenta versus placental and non-placental tumor cells*

### 6.1.1 *Human (h) chorionic somatomammotropin (CS) and growth hormone variant (GH-V) expression in normal and complicated pregnancies*

*Human* CS was isolated by Josimovitch and MacLaren in 1962 (Josimovich and Maclaren 1962), and is readily detected in the syncytiotrophoblasts but not trophoblasts of normal placenta by immunofluorescence (Heyderman, Gibbons et al. 1981). The concentration of *h*CS in maternal serum increases over the gestational period in normal intrauterine pregnancy (Mueller, Raio et al. 2004). Pregnancy-specific *h*GH-V expressed by syncytiotrophoblasts (Liebhaber, Urbanek et al. 1989) is detected in maternal plasma starting at 5 weeks of gestation (Chellakooty, Vangsgaard et al. 2004) and increases throughout pregnancy until term (Chellakooty, Skibsted et al. 2002). *Human* CS and *h*GH-V transcripts act as potential clinical diagnostic markers for progression of normal pregnancies as they are often decreased in pregnancies complicated by hypertension and maternal obesity (Farina, Sekizawa et al. 2006, Mannik, Vaas et al. 2012, Vakili, Jin et al. 2013). As discussed in Section *3.1, hCS-A, hCS-B and hGH-V* have differential expression profiles in normal pregnancies. Pregnancies complicated by placenta previa and invasive placenta are marked by increased *h*CS RNA transcripts (Kawashima, Sekizawa et al. 2014) and increased *hGH-V* expression is reported in gestational diabetic pregnancies with large-for-gestational-age

newborns (LGA) (Mannik, Vaas et al. 2012).

During pregnancy extravillous trophoblasts invade into uterine decidua and the inner third of the myometrium to achieve maternal-fetal exchange of molecules (Huppertz, Hemmings et al. 2005). Following these adaptive changes during pregnancy, extravillous trophoblast cells aberrantly retained in the uterus may become susceptible to formation of gestational choriocarcinoma (Huppertz, Hemmings et al. 2005). Gestational choriocarcinoma occurs with an approximate frequency of 1 in 40,000 pregnancies (Ganapathi, Paczos et al. 2010) and is often preceded by hydatidiform mole, ectopic pregnancy or spontaneous abortion (Kobayashi, Shimizu et al. 2011). Gestational trophoblastic choriocarcinoma and hydatidiform mole are documented with an extremely high chorionic gonadotropin (*h*CG) level (Mock, Kovalevskaya et al. 2000, Hsieh, Hsu et al. 2008) and a decrease in *h*CS (Mochizuki, Morikawa et al. 1976, Hoshina, Hussa et al. 1984, Nickel and Cattini 1991, Lytras, Bock et al. 1994).

### 6.1.2 *Altered human (h) chorionic somatomammotropin (CS) and growth hormone variant (GH-V) expression in placental choriocarcinoma and non-placental tumor cells*

In light of difficulty in establishing and maintaining primary placental cultures over a period of time efforts were made to establish human placental tumor (choriocarcinoma) cell lines such as BeWo, JAR, and JEG-3 cells (Ringler and Strauss 1990). Patillo and co-workers established the first hormone producing cell line BeWo. This choriocarcinoma cell line was derived from a choriocarcinoma serially transplanted by Hertz and colleagues in the hamster cheek pouch (Pattillo and Gey 1968, Pattillo, Gey et al. 1968). Patillo *et al* isolated colonies of tumor cells from explants and passed the cells until pure culture was obtained (Pattillo and Gey 1968).

Assessment by light and electron microscopy revealed that BeWo cells retained the cytotrophoblast characteristics of the original tumor, although rare differentiation to a syncytium can be observed (Ringler and Strauss 1990). Kohler and associates established the first clonally derived hormone producing choriocarcinoma cell lines including JEG-3 cells (Kohler and Bridson 1971, Kohler, Bridson et al. 1971). The JAR cell line, in contrast to JEG-3 and BeWo cells, was the first cell line to be established directly from a biopsy of choriocarcinoma occurring in a 24 year-old Caucasian female (Pattillo, Hussa et al. 1971). All the choriocarcinoma cell lines were reported to produce *h*CS (Ringler and Strauss 1990). Expression of *h*CS-A, *h*CS-B and *h*GH-V in all choriocarcinoma cell lines make it a convenient model to study *h*CS and *h*GH-V gene regulation (Nickel and Cattini 1991, Lytras, Bock et al. 1994). In addition to the choriocarcinoma cells, studies have also demonstrated ectopic *h*GH/CS gene expression in non-placental endometrial carcinoma cell lines (Khorram, Garthwaite et al. 2001, Sbracia, Scarpellini et al. 2004, Pandey, Perry et al. 2008) and adenocarcinoma cells such as the breast cancer MCF-7 cell line (Tuttle, Hugo et al. 2014). However, the expression profile of the individual *h*CS-A, *h*CS-B and *h*GH-V transcripts in tumor cells of non-placental origin have not been detected.

Relatively low levels of *h*GH/CS transcripts were observed in three placental and four of five non-placental tumor cell lines (Figure 3.1A) as assessed by RT-PCR, gel electrophoresis and ethidium bromide staining using primer sets that non-selectively amplify all the members of the *h*GH/CS gene family. This result is consistent with previous studies where RT-PCR or RNA blotting have been used (Nickel and Cattini 1991, Nachtigal, Bock et al. 1992, Lytras, Bock et al. 1994, Untergasser, Kranewitter et al. 1997, Tuttle, Hugo et al. 2014).

As discussed in Section ***3.1***, *h*CS and GH-V follows a differential pattern of expression in

normal uncomplicated pregnancies (MacLeod, Lee et al. 1992, Chen, Liao et al. 1989). Previous studies have employed RT-PCR using primer sets for each individual transcripts (MacLeod, Lee et al. 1992) or RT-PCR using a common primer set, followed by restriction digestion to distinguish *h*CS-A, *h*CS-B and *h*GH-V (Lytras, Bock et al. 1994). Our assessment here was extended to include individual *h*CS-A, *h*CS-B and *h*GH-V RNAs in normal term placenta, placental BeWo, JAR, JEG-3 *versus* non-placental HeLa, MCF-7 and U-87 tumor cells by qPCR for the very first time using specific primer sets. The results are consistent with previous findings, which support low levels of expression in tumor cells *versus* normal human term placenta, and are also consistent with BeWo and JAR in contrast to JEG-3 displaying preferential placental expression of individual *h*CS/GH-V genes (Nickel and Cattini 1991, Lytras and Cattini 1994). BeWo, JAR and JEG-3 are the most intensively studied choriocarcinoma cell lines along with a number of human-placenta-derived cell that have been used to model syncytiotrophoblast function (Sullivan 2004). The total *h*CS/GH-V RNA expression was 10-fold higher in BeWo cells than JAR cells, while it was undetectable or extremely low in JEG-3 cells as determined previously by methods such as cDNA cloning and RNA blotting (Nickel and Cattini 1991, MacLeod, Lee et al. 1992, Nachtigal, Nickel et al. 1993). The differences in patterns of expression of placental *h*CS/GH-V among choriocarcinoma cells suggest that they represent distinct populations of placental cells reflecting the heterogeneous nature of human placental cells available during the cytotrophoblast to syncytiotrophoblast transition in a deregulated trophoblastic system (Hochberg, Rachmilewitz et al. 1992, Lytras, Bock et al. 1994).

Interestingly, low comparable levels of *h*CS and *h*GH-V transcripts were observed in placental JEG-3 and non-placental MCF-7 and U87 cells. Cell authentication was not conducted to ensure

the cell lines are true to their origin but as a first approximation, this low levels could suggest that the placental origin of the JEG-3 cells is no longer apparent in the JEG-3 cells used in this project. Either the JEG-3 cells lack necessary 'placental' transcription factors required for preferential and efficient *h*CS/GH-V gene expression, and/or if these factors are available, their access to appropriate regulatory regions in the *h*GH/CS locus is limited.

### 6.1.3 *Differential response of regulatory 3´-Enh regions associated with expression of hCS/GH-V genes in placental versus non-placental tumor cells*

Based on *in vitro* and transgenic mouse *in vivo* studies, multiple regulatory regions have been implicated in the efficient expression of the *h*CS/GH-V genes. These include the proximal promoter region and 263-bp (263P) fragment of a region known as P sequences located about 2 kb upstream of *h*CS-L, *h*CS-A, *h*GH-V and *h*CS-B genes (Nachtigal, Nickel et al. 1993), 3´-enhancer regions located 2 kb downstream of the *h*CS-A and *h*CS-B genes (Rogers, Sobnosky et al. 1986, Lytras, Bock et al. 1994). In addition to the local *cis*-elements, remote upstream nuclease HS sites have been identified in a LCR including HS-III and HS-V and the placenta-specific HS-IV (Jones, Monks et al. 1995). As discussed in Section 1.7 of this thesis, efficient expression of *h*CS/GH-V genes requires combined actions of the local *cis*-regulatory elements and the distal elements. Gene transfer assays using hybrid *h*CSp-Luc gene constructs with regulatory 263P and 241-3´-Enh sequences in JEG-3 cells suggest that the choriocarcinoma cell line ***can*** support placental *h*CS expression (Jacquemin, Oury et al. 1994, Lytras and Cattini 1994). It can be inferred that even though the transcriptional apparatus is present in JEG-3 cells

that the most likely reason to explain the low *h*CS/GH-V RNA levels is a lack of access of the locus to these *trans*-acting factors. As previously stated (see section ***1.6.2***) there have been multiple reports of ectopic expression of *h*CS including in human breast cancer cell lines such as MCF-7 cells (Tuttle, Hugo et al. 2014). A differential response between placental and non-placental cell lines was not observed following transfection of the 263-bp (263P) sequence construct (Figure 4). A modest ~1.5-fold increase in *h*CS promoter activity was seen in JEG-3 cells. The decrease in *h*CS promoter activity with 263P in BeWo cells was not expected based on the 'positive' effect of P sequences on pituitary *hGH-N* expression in the placenta of transgenic mice (Elefant, Su et al. 2000). In addition, a modest positive effect of P sequences was detected on *h*CS promoter activity in transfected JEG-3 cells (Nachtigal, Nickel et al. 1993). Nonetheless, positive or negative, these data are consistent with the capacity for P sequences to influence promoter activity in placental cells.

In terms of the *h*CS 3´-Enh element, a preferential stimulation of *h*CS promoter activity was seen in JEG-3 as well as BeWo cells but not non-placental MCF-7 and U-87 cells. This is consistent with JEG-3 cells possessing "placental cell" characteristics in spite the low levels of endogenous hCS/GH-V RNA levels observed in this study, as well as the availability of *trans*-acting factors, in BeWo and JEG-3 cells, that can access the *h*CS 3´-Enh region. The enhancer region has presumably a relatively open (chromatin) configuration in the context of transfected plasmid DNA, but if the equivalent sequences cannot be accessed in the endogenous *h*CS/GH-V gene locus, this may explain the low levels of endogenous *hCS/GH-V* expression in tumor cells versus term placenta.

### *6.1.4 Differential effects of DNA demethylase and HDAC inhibition on hCS-A, hCS-B and hGH-V RNA levels*

Nucleosomes consist of a histone core around which ~146 bp of double helix DNA is wrapped twice (Kornberg and Lorch 1999). When these nucleosome units are linked together the resulting structure is termed *"chromatin"*. The condensation of this structure or regions of this structure can be controlled through various post-translational modifications of the core histone protein by reversible incorporation of, for example, acetyl groups within the N terminal tails of these proteins (Rothbart and Strahl 2014). DNA modifications can also play an active role in influencing chromatin structure (Henikoff, Furuyama et al. 2004, Ptashne 2005). An *open* state of chromatin allows transcription factors including RNA polymerase II to access the DNA and enhance RNA synthesis. DNA hypomethylation and histone hyperacetylation are features of an *open* chromatin structure (Eberharter and Becker 2002, Jaenisch and Bird 2003). DNA methylation marks integrate with histone modifications dynamically to regulate chromatin function (Cedar and Bergman 2009). Both DNA methylation and histone hyperacetylation have been linked to placental expression of the *h*GH/CS family (Hjelle, Phillips et al. 1982, Elefant, Su et al. 2000, Kimura, Liebhaber et al. 2004). Treatment with an inhibitor of histone deacetylation (TSA) resulted in an increase in *h*CS-A, *h*CS-B and *h*GH-V RNA levels in JEG-3 cells. Treatment with 25 µM azadC or greater resulted in modest but significant and consistent increases in *h*CS-B and *h*GH-V but not *h*CS-A RNA levels in JEG-3 cells. Differential response of *h*CS-A, *h*CS-B and *h*GH-V gene expression to histone deacetylation and DNA hypomethylation treatment suggest that DNA methylation and histone hyperacetylation alters

chromatin aceessibility at different regulatory regions. In spite of controlling chromatin accessibility by different mechanisms, there is evidence that DNA methylation may modulate local histone acetylation (Eden, Hashimshony et al. 1998).

### *6.1.5 Levels of hCS/GH-V transcripts are increased synergistically with sequential azadC and TSA but not TSA and azadC treatments in placental JEG-3 and not in non-placental MCF-7 cells*

Unmethylated DNA that contains acetylated histones is associated with open chromatin and increases accessibility of putative regulatory DNA elements (Weber and Schubeler 2007). By contrast, methylation of the same DNA sequences is associated with assembly of nucleosomes containing non-acetylated H3-H4 histones resulting in a more condensed chromatin configuration (Eden, Hashimshony et al. 1998, Hashimshony, Zhang et al. 2003, Weber and Schubeler 2007, Suzuki and Bird 2008).

The effect of sequential treatment with a DNA hypomethylating agent 50 µM azadC (24 hours) before inhibition of histone deacetylation 100 nM TSA (18 hours) on *h*CS/GH-V transcripts in JEG-3 and MCF-7 cells as assessed by qPCR resulted in a synergistic increase in JEG-3 cells. The synergistic effect with azadC and TSA treatment was not seen with non-placental MCF-7 cells. This synergistic response was specific to sequential azadC and TSA treatments in JEG-3 cells and was not observed upon reversal of the treatment. DNA and histone modifications function in a cooperative manner to modify chromatin organization and facilitate recruitment of effector proteins to regulatory locations throughout the genome (Cedar and Bergman 2009). Studies on derepression of the luteinizing hormone receptor (LHR) gene in choriocarcinoma JAR

cells suggest that treatment with HDAC inhibitor (TSA) evokes significant activation of the LHR gene, but maximal stimulation is achieved through the combined mechanistic actions of TSA in the continued presence of azadC (Zhang, Fatima et al. 2005). Failure to see the same synergistic increase in *h*CS/GH-V RNA levels when TSA was used before sequential treatment with azadC in JEG-3 cells suggest that stimulated expression of *h*CS/GH-V transcripts requires both hypomethylated DNA and hyperacetylated histones. As discussed in Section ***1.5,*** methylation sites are marked by methyl-CpG-binding proteins which recruit HDACs (repressors of transcription) (Nguyen, Gonzales et al. 2001). Pretreatment with azadC for 24 hours hypomethylates the DNA and blocks the recruitment of HDACs to the *h*GH/CS locus. Thus DNA hypomethylation boosts the recruitment of acetylase (activators of transcription). Post-treatment with azadC mutes the synergism response as the hypermethylated marks impedes recruitment of acetylase to the *h*GH/CS locus. TSA has also been shown to decrease global DNA methylation but in a manner distinct from azadC (Arzenani, Zade et al. 2011). The synergistic effect on *h*CS/GH-V transcripts seen in JEG-3 cells suggests that specific (azadC-related) demethylation provides a preferred basis for efficient histone hyperacetylation and sequence availability which can be achieved with prior azadC treatment enhancing the effects of TSA by interfering with the ability of methylcytosine – binding proteins to recruit HDACs to methylated regions (Jones, Veenstra et al. 1998, Nan, Ng et al. 1998, Wade, Jones et al. 1998, Cedar and Bergman 2009). We can interpret that DNA and histone modifications can make the chromatin more accessible and may affect binding of effector proteins that may read simultaneous modification states on both hypomethylated DNA and hyperacetylated histones through different interacting domains. For example: Mi-2/NuRD can sense hyper-methylated DNA through its MBD2 subunit and unmodified H3 N-terminal tails through its CHD4 and

CHD5 subunits (Musselman, Ramirez et al. 2012, Oliver, Musselman et al. 2012).

### *6.1.6 Significant increase in H3/H4 hyperacetylation at hCS 3´-Enh sequences, hCS-A/hGH-V promoters as well as HS III and the placenta–specific HS IV of the hGH/CS LCR*

Chromosomal remodeling is an important step, which may require the concerted effort of two distinct chromosomal remodeling activities: 1) histone acetyltransferase activity and, 2) ATP – driven chromosome – remodeling activity (Luo and Dean 1999). The question that follows is "*how are the histone acetyltransferases recruited to the DNA*"? Reports from yeast have indicated that usually transcription factor binding sites are nucleosome free (Grunstein 1990). It is possible that some transcription activators can readily bind to specific DNA sequences and recruit histone acetyltransferase complexes to the DNA (Defossez and Stancheva 2011). The recruitment of acetyltransferases acetylates histones across the genome and facilitates the binding of other transcription factors along with basal transcriptional machinery by making the sites more accessible (Bogdanovic and Veenstra 2009, Defossez and Stancheva 2011). Transcriptional repression has been shown with methylated DNA binding methyl CpG-binding domain (MBD) containing family (MeCP2). MeCP2 associate with multi-unit protein complexes containing histone deacetylases (HDACs). Transcriptional repression activity of MeCP2 is shown to be associated with the Sin3-HDAC and N-CoR-SMRT co-repressor complexes (Jones, Veenstra et al. 1998, Lyst, Ekiert et al. 2013). MBD proteins are known to be associated with nucleosome remodeling and histone deacetylase (NuRD) complexes (Le Guezennec, Vermeulen et al. 2006). Reporter luciferase assays have demonstrated that binding of the MBD proteins can lead to transcriptional repression (Ng, Zhang et al. 1999, Bakker, Lin et al. 2002, Jiang, Jin et al. 2002). Methyl CpG binding proteins was demonstrated to act as developmental stage-specific

transcriptional repressors in germ cell tumors and some somatic tissues (Jiang, Jin et al. 2002). Genome wide CpG hypermethylation is linked to gene silencing in various cell types (Kang, Bang et al. 1999, Dong, Kim et al. 2001) and treatment with azadC depletes DNMTs and reactivates transcription (Chiurazzi, Pomponi et al. 1998, Robert, Morin et al. 2003).

Studies done in this thesis raise the question of whether DNA methylation preceding histone acetylation plays a role in *h*GH/CS locus activation/expression *in vitro*? A cytosine residue can either be methylated or unmethylated. Enhancers tend to have fairly variable methylation status and their methylation status has been analyzed by whole - methylome analysis in plants and animals (Cokus, Feng et al. 2008, Lister, O'Malley et al. 2008, Lister, Pelizzola et al. 2009, Chodavarapu, Feng et al. 2010). Schmidl *et al.* found differentially methylated CpG region within the enhancers of subsets of T cells at various differentiation stages (Schmidl, Klug et al. 2009). The methylated CpG regions can result in reduced enhancer activity (Schmidl, Klug et al. 2009). Demethylation of CpG regions can activate the enhancer function (Wiench, John et al. 2011). The role of DNA methylation in the regulation *h*GH/CS locus has not been investigated. It remains unclear as to how DNA methylation affects *h*GH/CS expression. There is a question as to whether it directly increases accessibility of the chromatin or indirectly by controlling the binding of transcription factors.

The increased *h*CS/GH-V gene expression with sequential azadC and TSA treatments in JEG-3 cells was associated with significant increases in hyperacetylation at *h*CS 3´-Enh sequences, the *h*CS-A/*h*GH-V promoters as well as HS III and the placenta-specific HS IV of the *h*GH/CS LCR. Multiple transcription factors associated with these regulatory regions have been identified through gene transfer and/or *in situ* binding studies. Loss of C/EBPβ binding to the *h*CS 3´-Enh

sequences correlates with a decrease in *h*CS promoter activity with maternal obesity in pregnancy (Vakili, Jin et al. 2013). The CCAAT-enhancer-binding proteins (C/EBP) are a family of basic region / leucine zipper DNA binding proteins (Landschulz, Johnson et al. 1988). There are six transcription factors belonging to this family involved in tissue-specific metabolic gene transcription (Lekstrom-Himes and Xanthopoulos 1998). Investigation patterns of these six members have indicated that C/EBPβ has high expression levels in the syncytiotrophoblasts of the human placenta (Bamberger, Makrigiannakis et al. 2004). C/EBP family members, especially C/EBPβ was shown to recruit the chromatin remodeling complexes SWI/SNF (Kowenz-Leutz and Leutz 1999, Pedersen, Kowenz-Leutz et al. 2001). C/EBPβ has been shown to activate transcription by interaction with E1A-binding domain of p300 (Mink, Haenig et al. 1997). This suggests that C/EBPβ has the capacity at least in part to recruit a co-activator of transcription CBP/p300 (Chrivia, Kwok et al. 1993). CREB binding protein (CBP) and p300 is considered as a class of co-activators that binds to CREB transcription factors and can modify chromatin structure by recruiting histone acetyltransferases such as steroid receptor coactivator-1 (SRC-1), ACTR and p300/(CREB binding protein) associated factor (P/CAF) (Oelgeschlager, Janknecht et al. 1996, Mink, Haenig et al. 1997, Erickson, Hemati et al. 2001, Guo, Cichy et al. 2001, Duong, Waltner-Law et al. 2002, Jurado, Song et al. 2002). DNA elements for forkhead box F1 (FOXF1) site in the proximal *h*GH-V promoter region, and ETS-domain transcription factor family members in the HS III sequences of the LCR have been linked to efficient activity of the CS genes (Jin, Norquay et al. 2004, Lomenick, Hubert et al. 2006). Binding of activator protein-2 (AP-2) to HS III sequences correlates with histone H4 hyperacetylation in term placenta (Jin, Norquay et al. 2004) and CCCTC binding factor (CTCF) sites are present within the LCR and gene locus (Jin, Oomah et al. 2011, Tsai, Cooke et al. 2014). A possible explanation in the

increased *h*CS/GH-V gene expression with sequential azadC and TSA treatments in JEG-3 cells is the increased accessibility of the above mentioned transcription factors to the regulatory regions following an increase in histone hyper acetylation.

As discussed in Section 1.7, the local *cis*-regions and the distal LCR of the *h*GH/CS locus may work in conjunction for activated expression of *h*CS/GH-V genes. ChIP data suggest that there is a significant increase in histone H3-H4 hyperacetylation status at HS-IV after sequential azadC and TSA treatment. CTCF is recruited to the placenta specific HS-IV in two choriocarcinoma cell lines JEG-3 and BeWo as well as transgenic mice carrying 123-kb *h*GH/BAC transgene (Tsai, Cooke et al. 2014). CTCF and associated cohesin contribute differentially to chromatin architecture (Zuin, Dixon et al. 2014). It has been recently demonstrated that depletion of CTCF reduces intradomain interactions but leads to increased interaction with neighboring domains (Zuin, Dixon et al. 2014). CTCF is stably bound to the chromatin (Li, Huang et al. 2013) and maintain chromatin boundaries by dictating the recruitment of cohesin (Zuin, Dixon et al. 2014). Without CTCF binding cohesin will fail to localize appropriately and will form non-specific interactions reaching beyond boundaries (Zuin, Dixon et al. 2014). Recent studies suggests that loss of cohesin may result in loss of contact with neighboring enhancer sequences (Zuin, Dixon et al. 2014). DNA methylation blocks the binding of CTCF (Bell and Felsenfeld 2000). CTCF binds to demethylated DNA and can demethylate the neighboring regions (Stadler, Murr et al. 2011). This can increase the accessibility of the *h*CS/GH-V locus to the available transcription factors.

Observations made in this study suggest that accessibility of local and distal regulatory regions linked to stimulated placental expression are restricted by DNA methylation and histone

acetylation status of the *h*GH/CS locus in the JEG-3 cells. Our transfection data with hybrid luciferase genes containing *h*CS 3´-Enh sequences indicates the availability of transcription factors required for preferential enhancer activity in human choriocarcinoma cells. The inaccessibility of preferred regulatory regions presumably contributes to the low levels of *h*CS-A, *h*CS-B and *h*GH-V gene expression in placental tumor cells. Results described in Section **5.2** ***F & G*** indicate that the non-placental MCF-7 cells may require either higher doses of azadC and TSA or longer exposure to see a significant effect. This is in contrast to the placental JEG-3 cells, which responded with greater sensitively to the same treatment regimen. Thus, the non-responsiveness of the MCF-7 cells may be due to an inaccessible chromatin in the *h*GH/CS locus or, as indicated by our gene transfer data or lack available transcription factors to bind these regulatory regions and enhance transcription.

In summary, efficient *h*CS/GH-V gene expression requires interaction between local and remote regulatory regions *in vivo,* to facilitate appropriate access to available and necessary transcription factors. These regions are modified in terms of DNA methylation and histone acetylation status in human choriocarcinoma JEG-3 cells. These studies suggest that DNA demethylation followed by inhibition of histone deacetylation would offer an effective means to increase *h*CS/GH-V gene expression. The apparent increase in DNA accessibility of key regulatory regions, including 3´-Enh sequences, would then be able to take advantage of available placental enhancer transcription factors, required increased expression of the *h*CS/GH-V genes.

# CHAPTER 7

# FUTURE DIRECTIONS

Studies conducted in this thesis provide insight into the DNA methylation and histone acetylation status, and thus 'chromatin accessibility', at the *h*GH/CS locus in human placental choriocarcinoma JEG-3 cells. These studies can also provide some insight into the regulation of the *h*GH/CS locus *in vivo.* As discussed in Section 1.4, DNA methylation and histone acetylation regulate gene expression directly by changing chromatin accessibility or indirectly can change the whole cell methylation and acetylation status, which in turn can affect transcription factors availability. The experimental design used in this thesis needs modification if these studies were to be pursued further. To achieve significant demethylation, non-placental MCF-7 cells should be treated with higher doses or exposed longer to azadC. More importantly, while the studies done suggest increased accessibility, future studies would look at the availability and specific recruitment of transcription factors and its co-activators to the *h*GH/CS locus under conditions of TSA versus azadC and TSA treatments. Most important, studies would examine how does azadC and TSA treatments affect the chromatin architecture and the interactions (protein/protein and protein/DNA complexes formed) between local and remote regulatory sequences.

## *7.1 Mapping the methylated sites at the human (h) GH/CS locus*

Previous studies on the human GH/CS locus have looked into the acetylation status across the proximal and distal regulatory sequences as well as the locus control regions of the *h*GH/CS in term placenta (Kimura, Liebhaber et al. 2004, Kimura, Sizova et al. 2007). There is little available knowledge of DNA methylation status across the *h*GH/CS locus. DNA methylation has

been detected across the locus using methylation-sensitive enzyme assays but the technique lacked specificity as the individual hGH/CS genes share >90% sequence similarity (Hjelle, Phillips et al. 1982). Results in Section ***5.2 F & G***, indicate that function of DNA methylation is analogous to an amplifier and histone hyperacetylation presumably act as a switch to control transcription. I would suggest using a locus specific genome-wide array based approach such as immunoprecipitation or bisulfite pyro sequencing assay to map out the methylation sites (Shen and Waterland 2007). It will be useful to assess the methylation status across the *h*GH/CS locus under azadC versus DMSO vehicle controlled treatment conditions. Based on Figure ***5.3***, I expect to see a change in the DNA methylation status of the HS-IV, 3´-Enh and the *h*CS/GH-V promoter regions.

### *7.2* ***The transcription factors restricted from binding to methylated DNA***

It is unclear from the studies pursued in this thesis whether the change in gene expression is due to direct and/or indirect effects of azadC or TSA on the *h*CS/GH-V gene locus. For example, changes in the DNA methylation status directly affecting binding of (available) transcription factors to the *h*CS/GH-V locus or indirectly through effects on transcription factors and associated co-activators production (and thus binding to the locus). Binding of a few transcription factors like MYC is influenced by the methylation status of its cognate sequence (Prendergast and Ziff 1991) whereas SP1 is not influenced by methylation status (Harrington, Jones et al. 1988). Recent genome-wide studies have suggested that binding of transcription factors can be greatly influenced by methylation of CpG sites within its recognition sequence

(Chen, Feng et al. 2011). Interestingly not only the methylation of CpG sites within the transcription factor recognition sequence, but also within 100 bp away from the binding site can influence binding (Jones 2012). This observation was first reported in human embryonic stem cells, where octamer-binding transcription factor 4 (OCT4) was unable to bind to its cognate site when there was DNA methylation within 100 bp on either side of target sequence (You, Kelly et al. 2011, Jones 2012). As discussed in Section ***6.1.6***, methylated DNA can bind MDB proteins that in turn recruit HDACs and co-repressors, which can disrupt binding of transcription factors. It is of interest in the context of my thesis from my observations in figure ***5.2 F.*** In the *h*GH/CS locus it has been suggested that decreased binding of the transcription factor C/EBPβ leads to decreased expression of the *h*CS/GH-V genes (Vakili, Jin et al. 2013). If ***7.1*** is pursued, we will get to know if any CpG methylated sites exist within the C/EBPβ recognition site or the neighboring sequences. If yes, does it disrupt binding of C/EBPβ to the 3´-Enh regions and what makes C/EBPβ an important target? As mentioned in Section ***6.1.6***, these classes of transcription factors have the potential to recruit HATs and chromatin remodeling complexes to make the chromatin more accessible (Kowenz-Leutz and Leutz 1999, Pedersen, Kowenz-Leutz et al. 2001). Chromatin Immunoprecipitation techniques can be used to look into the binding of the transcription factor such as C/EBPβ. The recruitment of HATs to the ***h***GH/CS locus is not known. Discussed in Section ***1.6***, acetylation makes the chromatin accessible for transcription. For the *h*GH/CS locus recruitment of the HATs can help us understand whether stimulated *h*CS/GH-V expression requires opening of the entire *h*GH/CS locus in addition to chromosomal interactions between *cis*-elements and regulatory sequences. HAT activity can be determined by histone acetyl-transferase assay (HAT assay) with immunopurified proteins (Valineva, Yang et

al. 2005).

### *7.3 Histone methylation marks dictate DNA methylation status:*

Histone methylation has been extensively studied at the *h*GH/CS locus in conjunction with histone acetylation (Kimura, Liebhaber et al. 2004). The repressive trimethylation mark on histone H3 lysine (K) 9 residue [H3K9me3] can recruit HDACs (Veenstra and Wolffe 2001). As discussed in Section ***1.3***, acetylation can work in conjunction with other post-translational modifications and DNA methylation. Most importantly post-translational modifications and DNA methylation play an important role in the folding of chromatin structure that in turn regulates the differential expression pattern of these genes (Cedar and Bergman 2009).

My thesis has helped establish that placental JEG-3 cells have the necessary transcriptional apparatus for more increased *h*CS/GH-V expression. Histone acetylation and DNA methylation in the locus appear to limit chromatin accessibility, which accounts for the low levels of *h*CS/GH-V RNA detected relative to human term placenta. The role of DNA methylation in the regulation of the placental *h*CS/GH-V gene locus has been understudied, and my thesis has helped increase the potential importance of this DNA modification in the locus. Finally, these studies raise the possibility that combined and specific (given the sensitivity to the order of azadC and TSA treatments) effects on DNA methylation and histone acetylation may contribute to the repressed levels of *h*CS and *h*GH-V detected in choriocarcinoma versus term placenta *in vivo*.

# CHAPTER 8

# REFERENCES


Alberts, B., Bray D., Lewis J., Raff M., Roberts K., and Watson JD (1994). "Molecular biology of the cell." 3rd ed: Garland Publishing.

Anderson, J. D., P. T. Lowary and J. Widom (2001). "Effects of histone acetylation on the equilibrium accessibility of nucleosomal DNA target sites." J Mol Biol **307**(4): 977-985.

Arnaudo, A. M. and B. A. Garcia (2013). "Proteomic characterization of novel histone post-translational modifications." Epigenetics Chromatin **6**(1): 24.

Arzenani, M. K., A. E. Zade, Y. Ming, S. J. Vijverberg, Z. Zhang, Z. Khan, S. Sadique, L. Kallenbach, L. Hu, V. Vukojevic and T. J. Ekstrom (2011). "Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics." Mol Cell Biol **31**(19): 4119-4128.

Bakker, J., X. Lin and W. G. Nelson (2002). "Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells." J Biol Chem **277**(25): 22573-22580.

Bamberger, A. M., A. Makrigiannakis, M. Schroder, C. M. Bamberger, C. Relakis, B. Gellersen, K. Milde-Langosch and T. Loning (2004). "Expression pattern of the CCAAT/enhancer-binding proteins C/EBP-alpha, C/EBP-beta and C/EBP-delta in the human placenta." Virchows Arch **444**(2): 149-152.

Barrett, L. W., S. Fletcher and S. D. Wilton (2012). "Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements." Cell Mol Life Sci **69**(21): 3613-3634.

Barsh, G. S., P. H. Seeburg and R. E. Gelinas (1983). "The human growth hormone gene family: structure and evolution of the chromosomal locus." Nucleic Acids Res **11**(12): 3939-3958.

Bell, A. C. and G. Felsenfeld (2000). "Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene." Nature **405**(6785): 482-485.

Bell, O., V. K. Tiwari, N. H. Thoma and D. Schubeler (2011). "Determinants and dynamics of genome accessibility." Nat Rev Genet **12**(8): 554-564.

Bird, A. (2002). "DNA methylation patterns and epigenetic memory." Genes Dev **16**(1): 6-21.

Bogdanovic, O. and G. J. Veenstra (2009). "DNA methylation and methyl-CpG binding proteins: developmental requirements and function." Chromosoma **118**(5): 549-565.

Boime, I., D. McWilliams, E. Szczesna and M. Camel (1976). "Synthesis of human placental lactogen messenger RNA as a function of gestation." J Biol Chem **251**(3): 820-825.

Bowman, G. D. and M. G. Poirier (2015). "Post-translational modifications of histones that influence nucleosome dynamics." Chem Rev **115**(6): 2274-2295.

Braunstein, G. D., J. L. Rasor, E. Engvall and M. E. Wade (1980). "Interrelationships of human chorionic gonadotropin, human placental lactogen, and pregnancy-specific beta 1-glycoprotein

throughout normal human gestation." Am J Obstet Gynecol **138**(8): 1205-1213.

Brown, C. E., T. Lechner, L. Howe and J. L. Workman (2000). "The many HATs of transcription coactivators." Trends Biochem Sci **25**(1): 15-19.

Cattini, P. A., X. Yang, Y. Jin and K. A. Detillieux (2006). "Regulation of the human growth hormone gene family: possible role for Pit-1 in early stages of pituitary-specific expression and repression." Neuroendocrinology **83**(3-4): 145-153.

Cedar, H. (1988). "DNA methylation and gene activity." Cell **53**(1): 3-4.

Cedar, H. and Y. Bergman (2009). "Linking DNA methylation and histone modification: patterns and paradigms." Nat Rev Genet **10**(5): 295-304.

Chellakooty, M., L. Skibsted, S. O. Skouby, A. M. Andersson, J. H. Petersen, K. M. Main, N. E. Skakkebaek and A. Juul (2002). "Longitudinal study of serum placental GH in 455 normal pregnancies: correlation to gestational age, fetal gender, and weight." J Clin Endocrinol Metab **87**(6): 2734-2739.

Chellakooty, M., K. Vangsgaard, T. Larsen, T. Scheike, J. Falck-Larsen, J. Legarth, A. M. Andersson, K. M. Main, N. E. Skakkebaek and A. Juul (2004). "A longitudinal study of intrauterine growth and the placental growth hormone (GH)-insulin-like growth factor I axis in maternal circulation: association between placental GH and fetal growth." J Clin Endocrinol Metab **89**(1): 384-391.

Chen, E. Y., Y. C. Liao, D. H. Smith, H. A. Barrera-Saldana, R. E. Gelinas and P. H. Seeburg

(1989). "The human growth hormone locus: nucleotide sequence, biology, and evolution." Genomics **4**(4): 479-497.

Chen, P. Y., S. Feng, J. W. Joo, S. E. Jacobsen and M. Pellegrini (2011). "A comparative analysis of DNA methylation across human embryonic stem cell lines." Genome Biol **12**(7): R62.

Chiurazzi, P., M. G. Pomponi, R. Willemsen, B. A. Oostra and G. Neri (1998). "In vitro reactivation of the FMR1 gene involved in fragile X syndrome." Hum Mol Genet **7**(1): 109-113.

Chodavarapu, R. K., S. Feng, Y. V. Bernatavichute, P. Y. Chen, H. Stroud, Y. Yu, J. A. Hetzel, F. Kuo, J. Kim, S. J. Cokus, D. Casero, M. Bernal, P. Huijser, A. T. Clark, U. Kramer, S. S. Merchant, X. Zhang, S. E. Jacobsen and M. Pellegrini (2010). "Relationship between nucleosome positioning and DNA methylation." Nature **466**(7304): 388-392.

Chrivia, J. C., R. P. Kwok, N. Lamb, M. Hagiwara, M. R. Montminy and R. H. Goodman (1993). "Phosphorylated CREB binds specifically to the nuclear protein CBP." Nature **365**(6449): 855-859.

Cooke, N. E., J. Ray, M. A. Watson, P. A. Estes, B. A. Kuo and S. A. Liebhaber (1988). "Human growth hormone gene and the highly homologous growth hormone variant gene display different splicing patterns." J Clin Invest **82**(1): 270-275.

Defossez, P. A. and I. Stancheva (2011). "Biological functions of methyl-CpG-binding proteins." Prog Mol Biol Transl Sci **101**: 377-398.

Dom, G., V. C. Galdo, M. Tarabichi, G. Tomas, A. Hebrant, G. Andry, V. De Martelar, F. Libert, E. Leteurtre, J. E. Dumont, C. Maenhaut and W. C. van Staveren (2013). "5-aza-2'-deoxycytidine has minor effects on differentiation in human thyroid cancer cell lines, but modulates genes that are involved in adaptation in vitro." Thyroid **23**(3): 317-328.

Dong, S. M., H. S. Kim, S. H. Rha and D. Sidransky (2001). "Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix." Clin Cancer Res **7**(7): 1982-1986.

Duong, D. T., M. E. Waltner-Law, R. Sears, L. Sealy and D. K. Granner (2002). "Insulin inhibits hepatocellular glucose production by utilizing liver-enriched transcriptional inhibitory protein to disrupt the association of CREB-binding protein and RNA polymerase II with the phosphoenolpyruvate carboxykinase gene promoter." J Biol Chem **277**(35): 32234-32242.

Eberharter, A. and P. B. Becker (2002). "Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics." EMBO Rep **3**(3): 224-229.

Eden, S., T. Hashimshony, I. Keshet, H. Cedar and A. W. Thorne (1998). "DNA methylation models histone acetylation." Nature **394**(6696): 842.

Ehrlich, M. and K. C. Ehrlich (1993). "Effect of DNA methylation on the binding of vertebrate and plant proteins to DNA." EXS **64**: 145-168.

Elefant, F., Y. Su, S. A. Liebhaber and N. E. Cooke (2000). "Patterns of histone acetylation suggest dual pathways for gene activation by a bifunctional locus control region." EMBO J

**19**(24): 6814-6822.

Erickson, R. L., N. Hemati, S. E. Ross and O. A. MacDougald (2001). "p300 coactivates the adipogenic transcription factor CCAAT/enhancer-binding protein alpha." J Biol Chem **276**(19): 16348-16355.

Farina, A., A. Sekizawa, Y. Purwosunu, N. Rizzo, I. Banzola, M. Concu, D. Morano, F. Giommi, M. Bevini, M. Mabrook, P. Carinci and T. Okai (2006). "Quantitative distribution of a panel of circulating mRNA in preeclampsia versus controls." Prenat Diagn **26**(12): 1115-1120.

Felsenfeld, G. and M. Groudine (2003). "Controlling the double helix." Nature **421**(6921): 448-453.

Fitzpatrick, S. L., W. H. Walker and G. F. Saunders (1990). "DNA sequences involved in the transcriptional activation of a human placental lactogen gene." Mol Endocrinol **4**(12): 1815-1826.

Forsberg, E. C. and E. H. Bresnick (2001). "Histone acetylation beyond promoters: long-range acetylation patterns in the chromatin world." Bioessays **23**(9): 820-830.

Frankenne, F., J. Closset, F. Gomez, M. L. Scippo, J. Smal and G. Hennen (1988). "The physiology of growth hormones (GHs) in pregnant women and partial characterization of the placental GH variant." J Clin Endocrinol Metab **66**(6): 1171-1180.

Frenster, J. H., V. G. Allfrey and A. E. Mirsky (1963). "Repressed and Active Chromatin Isolated from Interphase Lymphocytes." Proc Natl Acad Sci U S A **50**: 1026-1032.

Garcia-Ramirez, M., C. Rocchini and J. Ausio (1995). "Modulation of chromatin folding by histone acetylation." J Biol Chem **270**(30): 17923-17928.

Glozak, M. A., N. Sengupta, X. Zhang and E. Seto (2005). "Acetylation and deacetylation of non-histone proteins." Gene **363**: 15-23.

Grunstein, M. (1990). "Histone function in transcription." Annu Rev Cell Biol **6**: 643-678.

Gudjonsson, J. E. and G. Krueger (2012). "A role for epigenetics in psoriasis: methylated Cytosine-Guanine sites differentiate lesional from nonlesional skin and from normal skin." J Invest Dermatol **132**(3 Pt 1): 506-508.

Guo, S., S. B. Cichy, X. He, Q. Yang, M. Ragland, A. K. Ghosh, P. F. Johnson and T. G. Unterman (2001). "Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPbeta." J Biol Chem **276**(11): 8516-8523.

Gurard-Levin, Z. A. and G. Almouzni (2014). "Histone modifications and a choice of variant: a language that helps the genome express itself." F1000Prime Rep **6**: 76.

Handwerger, S. (1991). "Clinical counterpoint: the physiology of placental lactogen in human pregnancy." Endocr Rev **12**(4): 329-336.

Harrington, M. A., P. A. Jones, M. Imagawa and M. Karin (1988). "Cytosine methylation does not affect binding of transcription factor Sp1." Proc Natl Acad Sci U S A **85**(7): 2066-2070.

Hashimshony, T., J. Zhang, I. Keshet, M. Bustin and H. Cedar (2003). "The role of DNA

methylation in setting up chromatin structure during development." Nat Genet **34**(2): 187-192.

Hattori, N., A. Shimatsu, M. Sugita, S. Kumagai and H. Imura (1990). "Immunoreactive growth hormone (GH) secretion by human lymphocytes: augmented release by exogenous GH." Biochem Biophys Res Commun **168**(2): 396-401.

Hebbes, T. R., A. L. Clayton, A. W. Thorne and C. Crane-Robinson (1994). "Core histone hyperacetylation co-maps with generalized DNase I sensitivity in the chicken beta-globin chromosomal domain." EMBO J **13**(8): 1823-1830.

Henikoff, S., T. Furuyama and K. Ahmad (2004). "Histone variants, nucleosome assembly and epigenetic inheritance." Trends Genet **20**(7): 320-326.

Hernando-Herraez, I., H. Heyn, M. Fernandez-Callejo, E. Vidal, H. Fernandez-Bellon, J. Prado-Martinez, A. J. Sharp, M. Esteller and T. Marques-Bonet (2015). "The interplay between DNA methylation and sequence divergence in recent human evolution." Nucleic Acids Res **43**(17): 8204-8214.

Heyderman, E., A. R. Gibbons and S. W. Rosen (1981). "Immunoperoxidase localisation of human placental lactogen: a marker for the placental origin of the giant cells in 'syncytial endometritis' of pregnancy." J Clin Pathol **34**(3): 303-307.

Hirt, H., J. Kimelman, M. J. Birnbaum, E. Y. Chen, P. H. Seeburg, N. L. Eberhardt and A. Barta (1987). "The human growth hormone gene locus: structure, evolution, and allelic variations." DNA **6**(1): 59-70.

Hjelle, B. L., J. A. Phillips, 3rd and P. H. Seeburg (1982). "Relative levels of methylation in human growth hormone and chorionic somatomammotropin genes in expressing and non-expressing tissues." Nucleic Acids Res **10**(11): 3459-3474.

Hochberg, A., J. Rachmilewitz, T. Eldar-Geva, T. Salant, T. Schneider and N. de Groot (1992). "Differentiation of choriocarcinoma cell line (JAr)." Cancer Res **52**(13): 3713-3717.

Holliday, R. and J. E. Pugh (1975). "DNA modification mechanisms and gene activity during development." Science **187**(4173): 226-232.

Hoshina, M., M. Boothby and I. Boime (1982). "Cytological localization of chorionic gonadotropin alpha and placental lactogen mRNAs during development of the human placenta." J Cell Biol **93**(1): 190-198.

Hoshina, M., M. Boothby, R. Hussa, R. Pattillo, H. M. Camel and I. Boime (1985). "Linkage of human chorionic gonadotrophin and placental lactogen biosynthesis to trophoblast differentiation and tumorigenesis." Placenta **6**(2): 163-172.

Hoshina, M., R. Hussa, R. Pattillo, H. M. Camel and I. Boime (1984). "The role of trophoblast differentiation in the control of the hCG and hPL genes." Adv Exp Med Biol **176**: 299-312.

Hsieh, T. Y., K. F. Hsu, P. L. Kuo and S. C. Huang (2008). "Uterine choriocarcinoma accompanied by an extremely high human chorionic gonadotropin level and thyrotoxicosis." J Obstet Gynaecol Res **34**(2): 274-278.

Hsu, J. Y., Z. W. Sun, X. Li, M. Reuben, K. Tatchell, D. K. Bishop, J. M. Grushcow, C. J.

Brame, J. A. Caldwell, D. F. Hunt, R. Lin, M. M. Smith and C. D. Allis (2000). "Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes." Cell **102**(3): 279-291.

Hsu, T. C. (1962). "Differential rate in RNA synthesis between euchromatin and heterochromatin." Exp Cell Res **27**: 332-334.

Huppertz, B., D. Hemmings, S. J. Renaud, J. N. Bulmer, P. Dash and L. W. Chamley (2005). "Extravillous trophoblast apoptosis--a workshop report." Placenta **26 Suppl A**: S46-48.

Imai, S., R. Kikuchi, H. Kusuhara, S. Yagi, K. Shiota and Y. Sugiyama (2009). "Analysis of DNA methylation and histone modification profiles of liver-specific transporters." Mol Pharmacol **75**(3): 568-576.

Jabbour, E., J. P. Issa, G. Garcia-Manero and H. Kantarjian (2008). "Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies." Cancer **112**(11): 2341-2351.

Jacquemin, P., E. Alsat, C. Oury, A. Belayew, M. Muller, D. Evain-Brion and J. A. Martial (1996). "The enhancers of the human placental lactogen B, A, and L genes: progressive activation during in vitro trophoblast differentiation and importance of the DF-3 element in determining their respective activities." DNA Cell Biol **15**(10): 845-854.

Jacquemin, P., C. Oury, B. Peers, A. Morin, A. Belayew and J. A. Martial (1994). "Characterization of a single strong tissue-specific enhancer downstream from the three human

genes encoding placental lactogen." Mol Cell Biol **14**(1): 93-103.

Jaenisch, R. and A. Bird (2003). "Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals." Nat Genet **33 Suppl**: 245-254.

Jenuwein, T. and C. D. Allis (2001). "Translating the histone code." Science **293**(5532): 1074-1080.

Jiang, C. L., S. G. Jin, D. H. Lee, Z. J. Lan, X. Xu, T. R. O'Connor, P. E. Szabo, J. R. Mann, A. J. Cooney and G. P. Pfeifer (2002). "MBD3L1 and MBD3L2, two new proteins homologous to the methyl-CpG-binding proteins MBD2 and MBD3: characterization of MBD3L1 as a testis-specific transcriptional repressor." Genomics **80**(6): 621-629.

Jin, Y., S. Y. Lu, A. Fresnoza, K. A. Detillieux, M. L. Duckworth and P. A. Cattini (2009). "Differential placental hormone gene expression during pregnancy in a transgenic mouse containing the human growth hormone/chorionic somatomammotropin locus." Placenta **30**(3): 226-235.

Jin, Y., L. D. Norquay, X. Yang, S. Gregoire and P. A. Cattini (2004). "Binding of AP-2 and ETS-domain family members is associated with enhancer activity in the hypersensitive site III region of the human growth hormone/chorionic somatomammotropin locus." Mol Endocrinol **18**(3): 574-587.

Jin, Y., K. Oomah and P. A. Cattini (2011). "Enhancer-blocking activity is associated with hypersensitive site V sequences in the human growth hormone locus control region." DNA Cell

Biol **30**(12): 995-1005.

Jones, B. K., B. R. Monks, S. A. Liebhaber and N. E. Cooke (1995). "The human growth hormone gene is regulated by a multicomponent locus control region." Mol Cell Biol **15**(12): 7010-7021.

Jones, P. A. (1999). "The DNA methylation paradox." Trends Genet **15**(1): 34-37.

Jones, P. A. (2012). "Functions of DNA methylation: islands, start sites, gene bodies and beyond." Nat Rev Genet **13**(7): 484-492.

Jones, P. A. and G. Liang (2009). "Rethinking how DNA methylation patterns are maintained." Nat Rev Genet **10**(11): 805-811.

Jones, P. L., G. J. Veenstra, P. A. Wade, D. Vermaak, S. U. Kass, N. Landsberger, J. Strouboulis and A. P. Wolffe (1998). "Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription." Nat Genet **19**(2): 187-191.

Josimovich, J. B. and J. A. Maclaren (1962). "Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone." Endocrinology **71**: 209-220.

Jurado, L. A., S. Song, W. J. Roesler and E. A. Park (2002). "Conserved amino acids within CCAAT enhancer-binding proteins (C/EBP(alpha) and beta) regulate phosphoenolpyruvate carboxykinase (PEPCK) gene expression." J Biol Chem **277**(31): 27606-27612.

Kang, S. H., Y. J. Bang, Y. H. Im, H. K. Yang, D. A. Lee, H. Y. Lee, H. S. Lee, N. K. Kim and

S. J. Kim (1999). "Transcriptional repression of the transforming growth factor-beta type I receptor gene by DNA methylation results in the development of TGF-beta resistance in human gastric cancer." Oncogene **18**(51): 7280-7286.

Kass, S. U., N. Landsberger and A. P. Wolffe (1997). "DNA methylation directs a time-dependent repression of transcription initiation." Curr Biol **7**(3): 157-165.

Kawashima, A., A. Sekizawa, W. Ventura, K. Koide, K. Hori, T. Okai, Y. Masashi, K. Furuya and Y. Mizumoto (2014). "Increased levels of cell-free human placental lactogen mRNA at 28-32 gestational weeks in plasma of pregnant women with placenta previa and invasive placenta." Reprod Sci **21**(2): 215-220.

Kelley, R. L. and M. I. Kuroda (2000). "The role of chromosomal RNAs in marking the X for dosage compensation." Curr Opin Genet Dev **10**(5): 555-561.

Kelly, T. K., T. B. Miranda, G. Liang, B. P. Berman, J. C. Lin, A. Tanay and P. A. Jones (2010). "H2A.Z maintenance during mitosis reveals nucleosome shifting on mitotically silenced genes." Mol Cell **39**(6): 901-911.

Khorram, O., M. Garthwaite, E. Grosen and T. Golos (2001). "Human uterine and ovarian expression of growth hormone-releasing hormone messenger RNA in benign and malignant gynecologic conditions." Fertil Steril **75**(1): 174-179.

Kimura, A. P., S. A. Liebhaber and N. E. Cooke (2004). "Epigenetic modifications at the human growth hormone locus predict distinct roles for histone acetylation and methylation in placental

gene activation." Mol Endocrinol **18**(4): 1018-1032.

Kimura, A. P., D. Sizova, S. Handwerger, N. E. Cooke and S. A. Liebhaber (2007). "Epigenetic activation of the human growth hormone gene cluster during placental cytotrophoblast differentiation." Mol Cell Biol **27**(18): 6555-6568.

Klassen, M. E., M. W. Nachtigal and P. A. Cattini (1989). "Human chorionic somatomammotropin gene expression in primary placental cell cultures." Placenta **10**(4): 321-329.

Koch, F. and J. C. Andrau (2011). "Initiating RNA polymerase II and TIPs as hallmarks of enhancer activity and tissue-specificity." Transcription **2**(6): 263-268.

Kohler, P. O. and W. E. Bridson (1971). "Isolation of hormone-producing clonal lines of human choriocarcinoma." J Clin Endocrinol Metab **32**(5): 683-687.

Kohler, P. O., W. E. Bridson, J. M. Hammond, B. Weintraub, M. A. Kirschner and D. H. Van Thiel (1971). "Clonal lines of human choriocarcinoma cells in culture." Acta Endocrinol Suppl (Copenh) **153**: 137-153.

Kornberg, R. D. (1974). "Chromatin structure: a repeating unit of histones and DNA." Science **184**(4139): 868-871.

Kornberg, R. D. and Y. Lorch (1999). "Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome." Cell **98**(3): 285-294.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.

Kowenz-Leutz, E. and A. Leutz (1999). "A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes." Mol Cell **4**(5): 735-743.

Krajewski, W. A. and P. B. Becker (1998). "Reconstitution of hyperacetylated, DNase I-sensitive chromatin characterized by high conformational flexibility of nucleosomal DNA." Proc Natl Acad Sci U S A **95**(4): 1540-1545.

Landschulz, W. H., P. F. Johnson and S. L. McKnight (1988). "The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins." Science **240**(4860): 1759-1764.

Latham, J. A. and S. Y. Dent (2007). "Cross-regulation of histone modifications." Nat Struct Mol Biol **14**(11): 1017-1024.

Le Guezennec, X., M. Vermeulen, A. B. Brinkman, W. A. Hoeijmakers, A. Cohen, E. Lasonder and H. G. Stunnenberg (2006). "MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties." Mol Cell Biol **26**(3): 843-851.

Lekstrom-Himes, J. and K. G. Xanthopoulos (1998). "Biological role of the CCAAT/enhancer-binding protein family of transcription factors." J Biol Chem **273**(44): 28545-28548.

Li, Y., W. Huang, L. Niu, D. M. Umbach, S. Covo and L. Li (2013). "Characterization of constitutive CTCF/cohesin loci: a possible role in establishing topological domains in mammalian genomes." BMC Genomics **14**: 553.

Liebhaber, S. A., M. Urbanek, J. Ray, R. S. Tuan and N. E. Cooke (1989). "Characterization and histologic localization of human growth hormone-variant gene expression in the placenta." J Clin Invest **83**(6): 1985-1991.

Liokatis, S., A. Stutzer, S. J. Elsasser, F. X. Theillet, R. Klingberg, B. van Rossum, D. Schwarzer, C. D. Allis, W. Fischle and P. Selenko (2012). "Phosphorylation of histone H3 Ser10 establishes a hierarchy for subsequent intramolecular modification events." Nat Struct Mol Biol **19**(8): 819-823.

Litt, M. D., M. Simpson, F. Recillas-Targa, M. N. Prioleau and G. Felsenfeld (2001). "Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci." EMBO J **20**(9): 2224-2235.

Lo, W. S., R. C. Trievel, J. R. Rojas, L. Duggan, J. Y. Hsu, C. D. Allis, R. Marmorstein and S. L. Berger (2000). "Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14." Mol Cell **5**(6): 917-926.

Lombardo, M. F., F. De Angelis, L. Bova, B. Bartolini, F. Bertuzzi, R. Nano, B. Capuani, R. Lauro, M. Federici, D. Lauro and G. Donadel (2011). "Human placental lactogen (hPL-A) activates signaling pathways linked to cell survival and improves insulin secretion in human pancreatic islets." Islets **3**(5): 250-258.

Lomenick, J. P., M. A. Hubert and S. Handwerger (2006). "Transcription factor FOXF1 regulates growth hormone variant gene expression." Am J Physiol Endocrinol Metab **291**(5): E947-951.

Luger, K., M. L. Dechassa and D. J. Tremethick (2012). "New insights into nucleosome and chromatin structure: an ordered state or a disordered affair?" Nat Rev Mol Cell Biol **13**(7): 436-447.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**(6648): 251-260.

Luo, R. X. and D. C. Dean (1999). "Chromatin remodeling and transcriptional regulation." J Natl Cancer Inst **91**(15): 1288-1294.

Lyst, M. J., R. Ekiert, D. H. Ebert, C. Merusi, J. Nowak, J. Selfridge, J. Guy, N. R. Kastan, N. D. Robinson, F. de Lima Alves, J. Rappsilber, M. E. Greenberg and A. Bird (2013). "Rett syndrome mutations abolish the interaction of MeCP2 with the NCoR/SMRT co-repressor." Nat Neurosci **16**(7): 898-902.

Lytras, A., M. E. Bock, J. G. Dodd and P. A. Cattini (1994). "Detection of placental growth hormone variant and chorionic somatomammotropin ribonucleic acid expression in human trophoblastic neoplasms by reverse transcriptase-polymerase chain reaction." Endocrinology **134**(6): 2461-2467.

Lytras, A. and P. A. Cattini (1994). "Human chorionic somatomammotropin gene enhancer activity is dependent on the blockade of a repressor mechanism." Mol Endocrinol **8**(4): 478-489.

Lytras, A., K. Detillieux and P. A. Cattini (2011). "Identification of functional CCAAT/enhancer-binding protein and Ets protein binding sites in the human chorionic

somatomammotropin enhancer sequences." J Mol Endocrinol **47**(2): 179-193.

Lytras, A., R. M. Surabhi, J. F. Zhang, Y. Jin and P. A. Cattini (1996). ""Repair' of the chorionic somatomammotropin-A "enhancer' region reveals a novel functional element in the chorionic somatomammotropin-B enhancer." Mol Cell Endocrinol **119**(1): 1-10.

MacLeod, J. N., A. K. Lee, S. A. Liebhaber and N. E. Cooke (1992). "Developmental control and alternative splicing of the placentally expressed transcripts from the human growth hormone gene cluster." J Biol Chem **267**(20): 14219-14226.

Mannik, J., P. Vaas, K. Rull, P. Teesalu and M. Laan (2012). "Differential placental expression profile of human Growth Hormone/Chorionic Somatomammotropin genes in pregnancies with pre-eclampsia and gestational diabetes mellitus." Mol Cell Endocrinol **355**(1): 180-187.

Mannik, J., P. Vaas, K. Rull, P. Teesalu, T. Rebane and M. Laan (2010). "Differential expression profile of growth hormone/chorionic somatomammotropin genes in placenta of small- and large-for-gestational-age newborns." J Clin Endocrinol Metab **95**(5): 2433-2442.

Matthias, P., M. Yoshida and S. Khochbin (2008). "HDAC6 a new cellular stress surveillance factor." Cell Cycle **7**(1): 7-10.

McWilliams, D. and I. Boime (1980). "Cytological localization of placental lactogen messenger ribonucleic acid in syncytiotrophoblast layers of human placenta." Endocrinology **107**(3): 761-765.

McWilliams, D., R. C. Callahan and I. Boime (1977). "Human placental lactogen mRNA and its

structural genes during pregnancy: quantitation with a complementary DNA." Proc Natl Acad Sci U S A **74**(3): 1024-1027.

Mersfelder, E. L. and M. R. Parthun (2006). "The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure." Nucleic Acids Res **34**(9): 2653-2662.

Mink, S., B. Haenig and K. H. Klempnauer (1997). "Interaction and functional collaboration of p300 and C/EBPbeta." Mol Cell Biol **17**(11): 6609-6617.

Mochizuki, M., H. Morikawa, K. Kawaguchi and S. Tojo (1976). "Growth hormone, prolactin and chorionic somatomammotropin in normal and molar pregnancy." J Clin Endocrinol Metab **43**(3): 614-621.

Mock, P., G. Kovalevskaya, J. F. O'Connor and A. Campana (2000). "Choriocarcinoma-like human chorionic gonadotrophin (HCG) and HCG bioactivity during the first trimester of pregnancy." Hum Reprod **15**(10): 2209-2214.

Mol, J. A., S. C. Henzen-Logmans, P. Hageman, W. Misdorp, M. A. Blankenstein and A. Rijnberk (1995). "Expression of the gene encoding growth hormone in the human mammary gland." J Clin Endocrinol Metab **80**(10): 3094-3096.

Morrish, D. W., L. H. Honore and D. Bhardwaj (1992). "Partial hydatidiform moles have impaired differentiated function (human chorionic gonadotropin and human placental lactogen secretion) in response to epidermal growth factor and 8-bromo-cyclic adenosine monophosphate." Am J Obstet Gynecol **166**(1 Pt 1): 160-166.

Mueller, M. D., L. Raio, S. Spoerri, F. Ghezzi, E. Dreher and N. A. Bersinger (2004). "Novel placental and nonplacental serum markers in ectopic versus normal intrauterine pregnancy." Fertil Steril **81**(4): 1106-1111.

Nachtigal, M. W., M. E. Bock, B. A. Kowaluk and P. A. Cattini (1992). "Chorionic gonadotrophin and c-myc expression in growing and growth-inhibited (intermediate) trophoblasts." Placenta **13**(4): 371-383.

Nachtigal, M. W., B. E. Nickel and P. A. Cattini (1993). "Pituitary-specific repression of placental members of the human growth hormone gene family. A possible mechanism for locus regulation." J Biol Chem **268**(12): 8473-8479.

Nan, X., H. H. Ng, C. A. Johnson, C. D. Laherty, B. M. Turner, R. N. Eisenman and A. Bird (1998). "Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex." Nature **393**(6683): 386-389.

Ng, H. H., Y. Zhang, B. Hendrich, C. A. Johnson, B. M. Turner, H. Erdjument-Bromage, P. Tempst, D. Reinberg and A. Bird (1999). "MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex." Nat Genet **23**(1): 58-61.

Nguyen, C. T., F. A. Gonzales and P. A. Jones (2001). "Altered chromatin structure associated with methylation-induced gene silencing in cancer cells: correlation of accessibility, methylation, MeCP2 binding and acetylation." Nucleic Acids Res **29**(22): 4598-4606.

Nickel, B. E. and P. A. Cattini (1991). "Tissue-specific expression and thyroid hormone regulation of the endogenous placental growth hormone variant and chorionic

somatomammotropin genes in a human choriocarcinoma cell line." Endocrinology **128**(5): 2353-2359.

Nickel, B. E. and P. A. Cattini (1996). "Nuclease sensitivity of the human growth hormone-chorionic somatomammotropin locus in pituitary and placenta suggest different mechanisms for tissue-specific regulation." Mol Cell Endocrinol **118**(1-2): 155-162.

Norquay, L. D., Y. Jin, R. M. Surabhi, R. D. Gietz, N. Tanese and P. A. Cattini (2001). "A member of the nuclear factor-1 family is involved in the pituitary repression of the human placental growth hormone genes." Biochem J **354**(Pt 2): 387-395.

Norquay, L. D., X. Yang, Y. Jin, K. A. Detillieux and P. A. Cattini (2006). "Hepatocyte nuclear factor-3alpha binding at P sequences of the human growth hormone locus is associated with pituitary repressor function." Mol Endocrinol **20**(3): 598-607.

Norquay, L. D., X. Yang, P. Sheppard, S. Gregoire, J. G. Dodd, W. Reith and P. A. Cattini (2003). "RFX1 and NF-1 associate with P sequences of the human growth hormone locus in pituitary chromatin." Mol Endocrinol **17**(6): 1027-1038.

Oelgeschlager, M., R. Janknecht, J. Krieg, S. Schreek and B. Luscher (1996). "Interaction of the co-activator CBP with Myb proteins: effects on Myb-specific transactivation and on the cooperativity with NF-M." EMBO J **15**(11): 2771-2780.

Ooi, S. K., C. Qiu, E. Bernstein, K. Li, D. Jia, Z. Yang, H. Erdjument-Bromage, P. Tempst, S. P. Lin, C. D. Allis, X. Cheng and T. H. Bestor (2007). "DNMT3L connects unmethylated lysine 4

of histone H3 to de novo methylation of DNA." Nature **448**(7154): 714-717.

Pandey, V., J. K. Perry, K. M. Mohankumar, X. J. Kong, S. M. Liu, Z. S. Wu, M. D. Mitchell, T. Zhu and P. E. Lobie (2008). "Autocrine human growth hormone stimulates oncogenicity of endometrial carcinoma cells." Endocrinology **149**(8): 3909-3919.

Pattillo, R. A. and G. O. Gey (1968). "The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro." Cancer Res **28**(7): 1231-1236.

Pattillo, R. A., G. O. Gey, E. Delfs, W. Y. Huang, L. Hause, D. J. Garancis, M. Knoth, J. Amatruda, J. Bertino, H. G. Friesen and R. F. Mattingly (1971). "The hormone-synthesizing trophoblastic cell in vitro: a model for cancer research and placental hormone synthesis." Ann N Y Acad Sci **172**(10): 288-298.

Pattillo, R. A., G. O. Gey, E. Delfs and R. F. Mattingly (1968). "Human hormone production in vitro." Science **159**(3822): 1467-1469.

Pattillo, R. A., R. O. Hussa and J. C. Garancis (1971). "Glycogen metabolism in human hormone-producing trophoblastic cells in continuous culture. I. Regulation of glycogen metabolism by glucose." In Vitro **7**(2): 59-67.

Pedersen, T. A., E. Kowenz-Leutz, A. Leutz and C. Nerlov (2001). "Cooperation between C/EBPalpha TBP/TFIIB and SWI/SNF recruiting domains is required for adipocyte differentiation." Genes Dev **15**(23): 3208-3216.

Prendergast, G. C. and E. B. Ziff (1991). "Methylation-sensitive sequence-specific DNA binding

by the c-Myc basic region." Science **251**(4990): 186-189.

Ptashne, M. (2005). "Regulation of transcription: from lambda to eukaryotes." Trends Biochem Sci **30**(6): 275-279.

Raccurt, M., P. E. Lobie, E. Moudilou, T. Garcia-Caballero, L. Frappart, G. Morel and H. C. Mertani (2002). "High stromal and epithelial human gh gene expression is associated with proliferative disorders of the mammary gland." J Endocrinol **175**(2): 307-318.

Richardson, B. D., R. A. Langland, C. J. Bachurski, R. G. Richards, C. A. Kessler, Y. H. Cheng and S. Handwerger (2000). "Activator protein-2 regulates human placental lactogen gene expression." Mol Cell Endocrinol **160**(1-2): 183-192.

Riggs, A. D. (1975). "X inactivation, differentiation, and DNA methylation." Cytogenet Cell Genet **14**(1): 9-25.

Ringler, G. E. and J. F. Strauss, 3rd (1990). "In vitro systems for the study of human placental endocrine function." Endocr Rev **11**(1): 105-123.

Robert, M. F., S. Morin, N. Beaulieu, F. Gauthier, I. C. Chute, A. Barsalou and A. R. MacLeod (2003). "DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells." Nat Genet **33**(1): 61-65.

Rogers, B. L., M. G. Sobnosky and G. F. Saunders (1986). "Transcriptional enhancer within the human placental lactogen and growth hormone multigene cluster." Nucleic Acids Res **14**(19): 7647-7659.

Rothbart, S. B. and B. D. Strahl (2014). "Interpreting the language of histone and DNA modifications." Biochim Biophys Acta **1839**(8): 627-643.

Sadakierska-Chudy, A. and M. Filip (2015). "A comprehensive view of the epigenetic landscape. Part II: Histone post-translational modification, nucleosome level, and chromatin regulation by ncRNAs." Neurotox Res **27**(2): 172-197.

Sbracia, M., F. Scarpellini, R. Poverini, P. L. Alo, G. Rossi and U. Di Tondo (2004). "Immunohistochemical localization of the growth hormone in human endometrium and decidua." Am J Reprod Immunol **51**(2): 112-116.

Schmidl, C., M. Klug, T. J. Boeld, R. Andreesen, P. Hoffmann, M. Edinger and M. Rehli (2009). "Lineage-specific DNA methylation in T cells correlates with histone methylation and enhancer activity." Genome Res **19**(7): 1165-1174.

Schubeler, D., C. Francastel, D. M. Cimbora, A. Reik, D. I. Martin and M. Groudine (2000). "Nuclear localization and histone acetylation: a pathway for chromatin opening and transcriptional activation of the human beta-globin locus." Genes Dev **14**(8): 940-950.

Schubeler, D., M. Groudine and M. A. Bender (2001). "The murine beta-globin locus control region regulates the rate of transcription but not the hyperacetylation of histones at the active genes." Proc Natl Acad Sci U S A **98**(20): 11432-11437.

Schwarzler, P., G. Untergasser, M. Hermann, S. Dirnhofer, B. Abendstein, S. Madersbacher and P. Berger (1997). "Selective growth hormone/placental lactogen gene transcription and hormone production in pre- and postmenopausal human ovaries." J Clin Endocrinol Metab **82**(10): 3337-

3341.

Scippo, M. L., F. Frankenne, E. L. Hooghe-Peters, A. Igout, B. Velkeniers and G. Hennen (1993). "Syncytiotrophoblastic localization of the human growth hormone variant mRNA in the placenta." Mol Cell Endocrinol **92**(2): R7-13.

Sewack, G. F., T. W. Ellis and U. Hansen (2001). "Binding of TATA binding protein to a naturally positioned nucleosome is facilitated by histone acetylation." Mol Cell Biol **21**(4): 1404-1415.

Shen, L. and R. A. Waterland (2007). "Methods of DNA methylation analysis." Curr Opin Clin Nutr Metab Care **10**(5): 576-581.

Shiota, K. (2004). "DNA methylation profiles of CpG islands for cellular differentiation and development in mammals." Cytogenet Genome Res **105**(2-4): 325-334.

Sigismund, S., S. Confalonieri, A. Ciliberto, S. Polo, G. Scita and P. P. Di Fiore (2012). "Endocytosis and signaling: cell logistics shape the eukaryotic cell plan." Physiol Rev **92**(1): 273-366.

Silva, J. R., J. R. Figueiredo and R. van den Hurk (2009). "Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis." Theriogenology **71**(8): 1193-1208.

Slater, M., M. Cooper and C. R. Murphy (2006). "Human growth hormone and interleukin-6 are upregulated in endometriosis and endometrioid adenocarcinoma." Acta Histochem **108**(1): 13-

18.

Stadler, M. B., R. Murr, L. Burger, R. Ivanek, F. Lienert, A. Scholer, E. van Nimwegen, C. Wirbelauer, E. J. Oakeley, D. Gaidatzis, V. K. Tiwari and D. Schubeler (2011). "DNA-binding factors shape the mouse methylome at distal regulatory regions." Nature **480**(7378): 490-495.

Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**(6765): 41-45.

Struhl, K. (1998). "Histone acetylation and transcriptional regulatory mechanisms." Genes Dev **12**(5): 599-606.

Sullivan, M. H. (2004). "Endocrine cell lines from the placenta." Mol Cell Endocrinol **228**(1-2): 103-119.

Suzuki, M. M. and A. Bird (2008). "DNA methylation landscapes: provocative insights from epigenomics." Nat Rev Genet **9**(6): 465-476.

Szulman, A. E. and U. Surti (1978). "The syndromes of hydatidiform mole. I. Cytogenetic and morphologic correlations." Am J Obstet Gynecol **131**(6): 665-671.

Takai, D. and P. A. Jones (2002). "Comprehensive analysis of CpG islands in human chromosomes 21 and 22." Proc Natl Acad Sci U S A **99**(6): 3740-3745.

Taverna, S. D., H. Li, A. J. Ruthenburg, C. D. Allis and D. J. Patel (2007). "How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers." Nat

Struct Mol Biol **14**(11): 1025-1040.

Tsai, Y. C., N. E. Cooke and S. A. Liebhaber (2014). "Tissue specific CTCF occupancy and boundary function at the human growth hormone locus." Nucleic Acids Res **42**(8): 4906-4921.

Tse, C., T. Sera, A. P. Wolffe and J. C. Hansen (1998). "Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III." Mol Cell Biol **18**(8): 4629-4638.

Tuttle, T. R., E. R. Hugo, W. S. Tong and N. Ben-Jonathan (2014). "Placental lactogen is expressed but is not translated into protein in breast cancer." PLoS One **9**(1): e87325.

Tyagi, S. (2015). "Tuning noise in gene expression." Mol Syst Biol **11**(5): 805.

Untergasser, G., W. Kranewitter, P. Schwarzler, S. Madersbacher, S. Dirnhofer and P. Berger (1997). "Organ-specific expression pattern of the human growth hormone/placental lactogen gene-cluster in the testis." Mol Cell Endocrinol **130**(1-2): 53-60.

Vakili, H., Y. Jin, S. Menticoglou and P. A. Cattini (2013). "CCAAT-enhancer-binding protein beta (C/EBPbeta) and downstream human placental growth hormone genes are targets for dysregulation in pregnancies complicated by maternal obesity." J Biol Chem **288**(31): 22849-22861.

Valineva, T., J. Yang, R. Palovuori and O. Silvennoinen (2005). "The transcriptional co-activator protein p100 recruits histone acetyltransferase activity to STAT6 and mediates interaction between the CREB-binding protein and STAT6." J Biol Chem **280**(15): 14989-14996.

Varala, K., Y. Li, A. Marshall-Colon, A. Para and G. M. Coruzzi (2015). ""Hit-and-Run" leaves its mark: catalyst transcription factors and chromatin modification." Bioessays **37**(8): 851-856.

Veenstra, G. J. and A. P. Wolffe (2001). "Constitutive genomic methylation during embryonic development of Xenopus." Biochim Biophys Acta **1521**(1-3): 39-44.

Venkatesh, S. and J. L. Workman (2015). "Histone exchange, chromatin structure and the regulation of transcription." Nat Rev Mol Cell Biol **16**(3): 178-189.

Vettese-Dadey, M., P. A. Grant, T. R. Hebbes, C. Crane- Robinson, C. D. Allis and J. L. Workman (1996). "Acetylation of histone H4 plays a primary role in enhancing transcription factor binding to nucleosomal DNA in vitro." EMBO J **15**(10): 2508-2518.

Vignali, M., D. J. Steger, K. E. Neely and J. L. Workman (2000). "Distribution of acetylated histones resulting from Gal4-VP16 recruitment of SAGA and NuA4 complexes." EMBO J **19**(11): 2629-2640.

Wade, P. A., A. Gegonne, P. L. Jones, E. Ballestar, F. Aubry and A. P. Wolffe (1999). "Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation." Nat Genet **23**(1): 62-66.

Wade, P. A., P. L. Jones, D. Vermaak, G. J. Veenstra, A. Imhof, T. Sera, C. Tse, H. Ge, Y. B. Shi, J. C. Hansen and A. P. Wolffe (1998). "Histone deacetylase directs the dominant silencing of transcription in chromatin: association with MeCP2 and the Mi-2 chromodomain SWI/SNF ATPase." Cold Spring Harb Symp Quant Biol **63**: 435-445.

Walker, W. H., S. L. Fitzpatrick and G. F. Saunders (1990). "Human placental lactogen transcriptional enhancer. Tissue specificity and binding with specific proteins." J Biol Chem **265**(22): 12940-12948.

Wang, X., S. C. Moore, M. Laszckzak and J. Ausio (2000). "Acetylation increases the alpha-helical content of the histone tails of the nucleosome." J Biol Chem **275**(45): 35013-35020.

Weber, M. and D. Schubeler (2007). "Genomic patterns of DNA methylation: targets and function of an epigenetic mark." Curr Opin Cell Biol **19**(3): 273-280.

Wittschieben, B. O., G. Otero, T. de Bizemont, J. Fellows, H. Erdjument-Bromage, R. Ohba, Y. Li, C. D. Allis, P. Tempst and J. Q. Svejstrup (1999). "A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme." Mol Cell **4**(1): 123-128.

Wolffe, A. P. (1998). "Packaging principle: how DNA methylation and histone acetylation control the transcriptional activity of chromatin." J Exp Zool **282**(1-2): 239-244.

Wolffe, A. P. and J. J. Hayes (1999). "Chromatin disruption and modification." Nucleic Acids Res **27**(3): 711-720.

Wu, H., R. Devi and W. B. Malarkey (1996). "Localization of growth hormone messenger ribonucleic acid in the human immune system--a Clinical Research Center study." J Clin Endocrinol Metab **81**(3): 1278-1282.

Yagi, S., K. Hirabayashi, S. Sato, W. Li, Y. Takahashi, T. Hirakawa, G. Wu, N. Hattori, N. Hattori, J. Ohgane, S. Tanaka, X. S. Liu and K. Shiota (2008). "DNA methylation profile of

tissue-dependent and differentially methylated regions (T-DMRs) in mouse promoter regions demonstrating tissue-specific gene expression." Genome Res **18**(12): 1969-1978.

Yang, X. J. and E. Seto (2008). "The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men." Nat Rev Mol Cell Biol **9**(3): 206-218.

You, J. S., T. K. Kelly, D. D. De Carvalho, P. C. Taberlay, G. Liang and P. A. Jones (2011). "OCT4 establishes and maintains nucleosome-depleted regions that provide additional layers of epigenetic regulation of its target genes." Proc Natl Acad Sci U S A **108**(35): 14497-14502.

Zhang, K. and H. Tang (2003). "Analysis of core histones by liquid chromatography-mass spectrometry and peptide mapping." J Chromatogr B Analyt Technol Biomed Life Sci **783**(1): 173-179.

Zhang, Y., N. Fatima and M. L. Dufau (2005). "Coordinated changes in DNA methylation and histone modifications regulate silencing/derepression of luteinizing hormone receptor gene transcription." Mol Cell Biol **25**(18): 7929-7939.

Zhou, V. W., A. Goren and B. E. Bernstein (2011). "Charting histone modifications and the functional organization of mammalian genomes." Nat Rev Genet **12**(1): 7-18.

Zuin, J., J. R. Dixon, M. I. van der Reijden, Z. Ye, P. Kolovos, R. W. Brouwer, M. P. van de Corput, H. J. van de Werken, T. A. Knoch, I. W. F. van, F. G. Grosveld, B. Ren and K. S. Wendt (2014). "Cohesin and CTCF differentially affect chromatin architecture and gene expression in human cells." Proc Natl Acad Sci U S A **111**(3): 996-1001.