Molecular Basis of GATA-4 Expression During the Early Commitment Stage of Cardiomyogenesis

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ABSTRACT

Cardiovascular diseases are among the leading causes of death in North America. Currently, there are no effective treatment options for directly repairing the damaged myocardial tissue. Therefore, cell-based therapies utilizing cardiomyocytes generated from stem cells to replace necrotic tissue will be a promising approach. However, the molecular mechanisms regulating stem cell differentiation into cardiomyocytes are not fully understood. Since GATA-4 is one of the primary regulators of cradiomyogenesis, we investigated the molecular basis of GATA-4 expression during the early stages of stem cell differentiation. Using chromatin immunoprecipitation, we have observed the direct involvement of p300 in GATA-4 gene expression. We have also examined the importance ofhHistone acetylation and acetyltransferase activity on GATA-4 expression during the early stage of cardiomyogensis using the histone deactylase inhibitor Valproic Acid and the acetyltransferase inhibitor Curcumin respectively.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANF	Atrial Natriuretic Factor
α-MEM	Alpha Minimum Essential Medium
β-MHC	β- Myosin Heavy Chain
BMP	Bone Morphogenetic Protein
BRCA-1	Breast Cancer-1 gene
cAMP	cyclic Adenosine Monophosphate
Cited2	CBP/p300-interacting ransactivator with glutamic acid (E)/aspartic acid (D)-rich tail
ChIP	Chromatin Immunoprecipitation
CPC	Cardiac Progenitor Cell
CREB	cAMP Response Element-Binding
CS	Calf Serum
Cx43	Connexin 43
DNA	Deoxyribonucleic acid
DKK1	Dickkopf-related protein 1
D-MEM	Dubelco-Minimum Essential Medium
Dnmt3a	DNA methyltransferase
DSP	Desmoplakin
EB	Embryoid Body
EC cell	Embryonic Carcinoma cell
ES cell	Embryonic Stem cell
FACS	Fluorescence-Activated Cell Sorting
FAT	Factor Acetyltransferase

FBS	Fetal Bovine Serum
FgF	Fibroblast growth Factor
GATA	GATA Binding Factor
GNAT	GCN5-related family of N-acetyltransferase
GSk3β	Glycogen Synthase 3β
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HES1	Hair and Enhancer of Split1
ILK	Integrin-Linked Kinase
Klf4	Krueppel-like Factor 4
LEF	Lymphoid Enhancer Factor
LIF	Leukemia Inhibitory Factor
MEF2C	Myocyte Enhancer Factor 2C
mRNA	messenger Ribonucleic acid
MyoD	Skeletal muscle specific gene
mTOR	mammalian Target of Rapamycin
MYST	Histone Acetyltransferase
NFAT	Nuclear Factor of Activated T-cells
NCID	Notch Intracellular Domain
Notch	Signaling cascade
Nppa	Natriuretic peptide precursor type A
Oct3/4	member of the POUF family of transcription factors
Oct-4	member of the POUF family of transcription factors

P19	Embryonic Carcinoma cell line
p53	Intracellular protein
p300	Histone Acetyltransferase
P/CAF	p300/CBP Associated Factor
PI3K/Akt	Phosphotidylinositol Kinase 3 pathway
PCR	Polymerase Chain Reaction
PTEN	Phosphatase and Tensin Homologue
RAR	Retinoic Acid Receptor
ROS	Reactive Oxygen Species
Shh	Sonic Hedgehog signaling
siRNA	splicing RNA
SOX2	Sex determining region Y-box 2
Stat3	Signal transducer and activator of transcription 3
SUMO	Ubiquitin-like modifier
SRF	Serum Response Factor
TATA	Transcription machinery docking domain
TCF	Transcription factor
TFIIB	Transcription Factor IIB
Tgf-β	Transforming Growth factor-β
VPA	Valproic acid
WGATAR	Sequence for GATA factor binding
Wnt	Signaling pathway

INTRODUCTION

Cardiovascular diseases are among the leading causes of death in North America and account for 29% of all deaths in Canada according to the latest report from Statistics Canada. (http://www.heartandstroke.com/site/c.ikIQLcMWJtE/b.3483991/k.34A8/Statistics.htm). Most survivors of cardiovascular diseases live with inadequate heart function (Lloyd-Jones et al., 2009). This is in part due to limited ability of human cardiomyocytes to proliferate and regenerate after birth, unlike other tissues in the body like skeletal muscle, liver, and skin which maintain high regenerative during adulthood ((Tedesco, Dellavalle, Diaz-Manera, Messina, & Cossu, 2010); (Schmucker & Sanchez, 2011); (Blanpain & Fuchs, 2009)). In comparison to these tissues, as well as the hearts of other species such as amphibians and zebrafish which have displayed recovery post tissue insult ((Ausoni & Sartore, 2009); (Chablais, Veit, Rainer, & Jazwinska, 2011)) the human heart triumphs as the least plastic.

Recent studies have shown the presence of pluripotent cells residing in the cardiac tissue itself, which was capable of being stimulated with Thymosin treatment and mobilizing to the site of injury. The new cells were able to integrate with the residing cells in areas of tissue damage and form connections that were the same electrically and in terms of contractility (Smart et al., 2011). Currently, there are clinical trials for bone marrow derived cells that are delivered post infarction, however, their therapeutic outcomes are modest and potentially have effects through chemical signaling as opposed to direct integration. Moreover, they are not pluripotent in origin (reviewed in(Laflamme & Murry, 2011)). In terms of using cardiomyocytes derived from pluripotent stem cells or from induced pluripotent stem cells, a number of obstacles remain to be

overcome including the risk of teratocarcinoma formation, viability, and efficiency of the differentiated cell harvest (Nussbaum et al., 2007).

Therefore, elucidating the molecular pathways involved in the gene cascade during the commitment of stem cells into the cardiac lineage is essential for developing techniques to generate large quantities of functional heart cells to be used in therapy.

Cardiac Response to Physiologic and Pathologic Insult

Therefore, when the heart is faced with an increased workload the heart tries to compensate to external increased workload (hemodynamic overload) by undergoing undivided myocyte growth (hypertrophy). Not all hypertrophy is pathologic though, as it is often seen in athletes, pregnant women, and developing children to some extent without any negative effects (Colan, 1997). However, myocyte growth also occurs in response to pressure overload from hypertension, valvular disease, myocardial infarction (MI), and cardiomyopathy. The sarcomeres are added in parallel to each other thereby thickening the myocytes and increasing the systolic pressure, thereby maintaining efficiency of the blood pumped out of the ventricles. In contrast, in chronic aortic regurgitation, mitral regurgitation, or anemia, the sarcomeres are added end to end, thereby increasing the length of the heart cells. This decreases the ventricle wall thickness to chamber dimension, and increases the stroke volume. In addition to this, the heart repairs the tissue damage incurred by an MI with fibroblasts. This results in the formation of scar tissue which results in the deposition of fibrous tissue into the healthy myocardium, and ventricle wall stiffness eventually impairing the overall contractility of the heart (Sun & Weber, 2000). In time, these compensatory mechanisms by the non-proliferative heart proves pathologic leading to

irregular electrical activity within the heart, congestive heart failure, and eventual death as the heart becomes less efficient (Lorell & Carabello, 2000).

Cardiac Development at Tissue Level

Heart is the first organ to become fully functional during embryonic development, and its contractile cells are called cardiomyocytes. During vertebral embryonic development the precursor cells (for both myocardium and endocardium) originate in the anterior lateral plate mesoderm, organized in bilateral fields around the rostral notochord and prechordal plate. The merging of these fields at the anterior margin gives rise to the cardiac crescent, which then comes together and merges along the midline forming the beating heart tube at murine embryonic day 8.5 (E 8.5) and corresponds to three weeks of gestation in human beings. Following the rightward looping of the heart tube guided by temporal and spatial signals within and around the heart tube; septation results in the formation of the four chambered heart as reviewed by (Zaffran & Frasch, 2002).

Effect of Post Translational Remodeling on Gene expression

In mammalian cells the DNA is in a compacted state with 146bp of DNA wrapped around a core octamer histone complex composed of a pair of H2A, H2B, H3, and H4 histones forming the chromatin. The chromatin is further folded and compressed by additional proteins like the linker H1 histones. Therefore, posttranslational modifications are important for regulating gene expression. For instance, the acetylation of specific lysine residues in the histone tails by HATs results in the neutralization of the positive charge. This disrupts the DNA-protein and protein-protein interactions, causing the chromatin to relax and increases access to transcription factors

and machinery. In contrast, HDACs promote condensation and thereby repress transcription most of the time. Moreover, the activity/DNA binding ability of various proteins that play a role in transcription is affected by their acetylation state. Specificity of HATs and HDACs is conferred by their association with DNA-binding factors (Allis & Wu, 2004).

Role of HDACs in Cardiac Development at a Cellular Level

HDACs are classified into 3 classes based on their homology with 3 distinct yeast HDACs. Class I and III HDACs are ubiquitously expressed; whereas, class II HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) are primarily expressed in the muscle, brain, and T-cells (Verdin, Dequiedt, & Kasler, 2003). Members of this group also have a large N-terminal domain for interaction with various transcriptional activators and repressors (Verdin et al., 2003). Among the class II HDACs, HDAC5 is the most abundant form expressed in the heart (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003) and HDAC9 play an important role in normal heart development and for repressing hypertrophy. For instance, most of the HDAC5/9 double-null animals die from retarded cardiac development, ventricular-septal malformation, and thin myocardial wall (Chang et al., 2004). In addition to this, mice lacking HDAC5 and HDAC9 develop cardiac hypertrophy at about 6 months, despite displaying normal cardiac function earlier (Zhang et al., 2002). HDAC5 and HDAC9 mutant mice develop hypertrophic hearts when they are exposed to pressure overload by aortic constriction (Molkentin, 2000). Class II HDAC levels doesn't change in response to hypertrophic stressors; however, their cellular localization is affected. In response to hypertrophic signals, CaMKII and PKD1 phosphorylate the 2 serine residues in the amino terminal results in interaction with the 14-3-3 chaperone which hides the nuclear localization signal, and exposes the nuclear export signal. The nuclear export signal is

bound by the Chromosome Region Maintenance1 (CRM1) nuclear export receptor, thereby shuttling the class II HDACs out of the nucleus, and allowing the transcription factors involved in the activation of the hypertrophic response to become activated by the removal of the repressor and possible HAT interaction (Grozinger & Schreiber, 2000) and (McKinsey, Zhang, & Olson, 2001).

Role of p300 and CBP in Cardiac Development at a Cellular Level

HATs include members of the GNAT family, MYST group, and p300/CBP family (X. J. Yang, 2004). Of these, the p300 and CREB-Binding Protein (CBP) are ubiquitously expressed nuclear phosphoproteins which are transcriptional co-activators that play a major role in cell cycle control, differentiation, and apoptosis (Giordano & Avantaggiati, 1999). The co-activators p300 and CBP where first brought into the scientific lime light from a developmental perspective after the observation of its role in Rubinstein-Taybi Syndrome. It was found that mutation of the CBP gene resulted in defective lung, kidney, eye, lung, skin, and testicle development, upper and lower limb digitation, as well as mental and overall growth retardation (Rubinstein, 1990). It was shown that p300 and CBP protein was present in mouse embryos and oocytes, and was nuclearized during embryonic development with both decreasing post-natally (Kalkhoven, 2004); (G. Chen, Zhu, Lv, Wu, Sun, Huang, & Tian, 2009b). However, an *in vivo* study by Partenen and colleagues (1999), revealed that both CBP and p300 were detected in murine cardiac tissue on embryonic day 8.5, high levels continuing to be detected in the atrial and ventricular myocytes during further development, followed by halting of expression by embryonic day 14.5 in the heart (Partanen, Motoyama, & Hui, 1999).

CBP and p300 co-activators are highly homologous sharing 63% of their amino acid sequence. They both have multiple protein interaction domains including the nuclear receptor interaction domain (66%) which binds RAR; CREB and MYB (KIX) domain (86%) binding CREB, BRCA-1, c-Jun and more; Cysteine and Histidine rich areas (C/H) (93% for C/H1), of which the C/H3 domain was shown to interact with P/CAF, RNA Helicase and TFIIB of the transcription machinery, GATA-1 and more; acetyltransferase and a bromodomain region (86%), with the percentage of overlap between p300 and CBP at these domains indicated in brackets at the amino acid level. (Giles, Peters, & Breuning, 1998). In addition to interacting with other proteins to regulate gene cascades, these regions are also involved in regulation of the co-activator itself. For instance, phosphorylation in the C-terminal of p300 correlated with increased HAT activity and p300 occupancy at promoters. The bromodomain was shown to be involved in the regulation of the co-activator p300 itself within the cell. Whereby, dephosphorylation of p300 resulted in ubiquitation of this domain and p300 degradation, while SUMOlaytion close to it decreased transcription (for a full review refer to Chen and Li, 2011).

The p300/CBP protein is thought to potentially function in the following ways (Figure 1); as a bridge between the basal transcriptional machinery and the transcription factors, as a scaffold for various factors, Histone Acetyltransferase (HAT), and as a Factor Acetyltransferase (FAT) (Chan & La Thangue, 2001). The diverse roles undertaken by these co-activators allow them to act as regulators, and integrators of multiple cellular pathways.

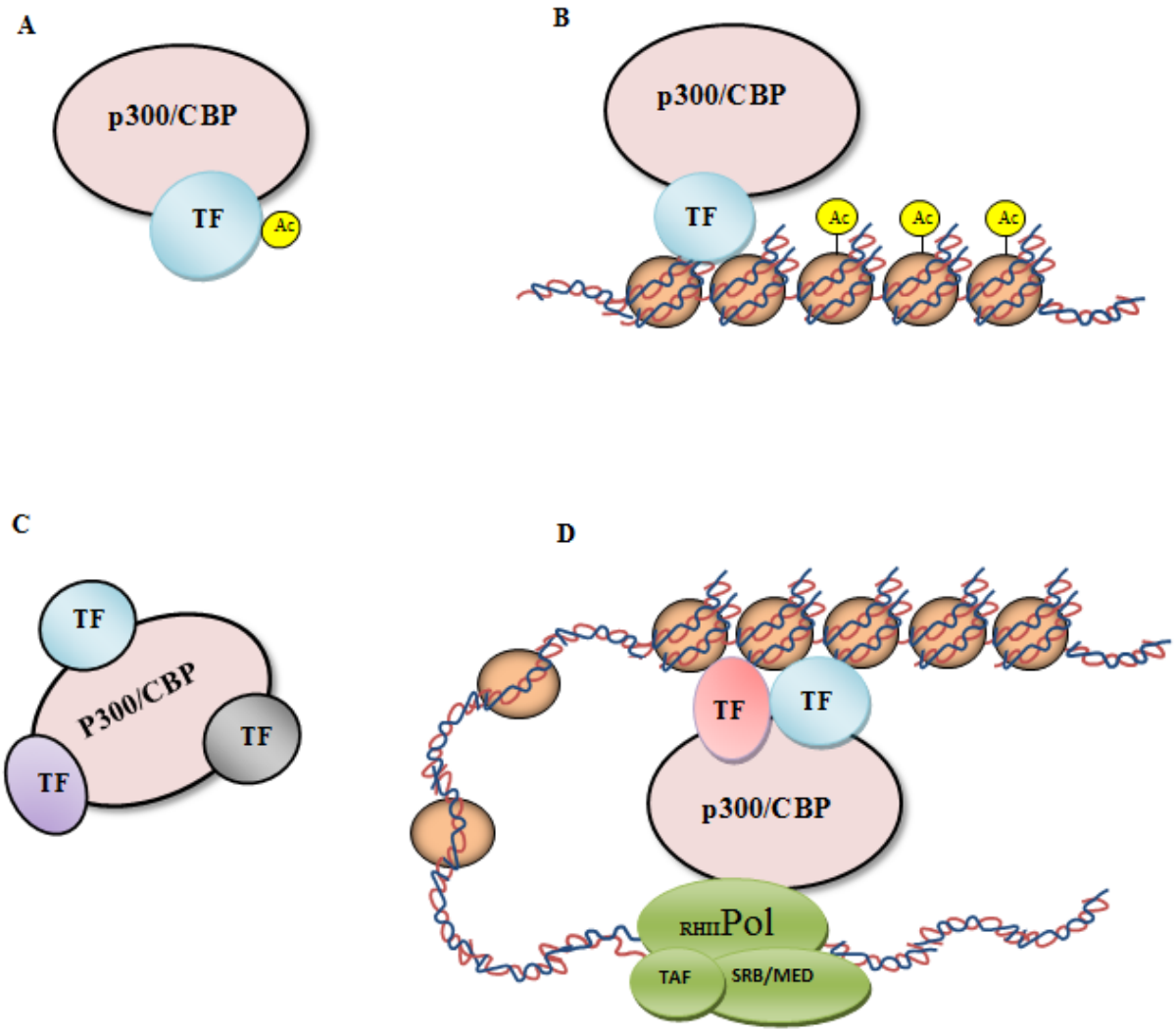

Figure 1. p300 and CBP function. Schematic showing the possible mechanisms of p300/CBP activity in the cell. p300/CBP have an acetyltransferase domain capable of acetylating (Ac) transcription factors (TF)(A) and histones (represented by the beads) (B). Additionally, p300/CBP can serve as a scaffold for various transcription factors (C), and as a bridge between the transcriptional machinery at the promoter and the transcription factors bound to enhancer regions upstream or downstream from the promoter (D).

Functional and Spatial differences between p300 and CBP during Cardiac Development

Despite their similarity, recent studies are indicating a functional difference between p300 and CBP. For instance, p300 was found to acetylate p53 and increase its DNA binding ability, causing the expression of genes involved in cell cycle arrest and apoptosis, hence its anti-tumor effects ((Gu et al., 1997; Gu, Shi, & Roeder, 1997)). In contrast, CBP was found to be more involved in transcription inhibition than p300 in a target gene analysis study (Ramos et al., 2010). Moreover, knockout of p300 or CBP lead to developmental defects, indicating that these proteins can't functionally compensate for each other (Kwok, Liu, & Smith, 2006).

In terms of cell specificity, p300 was found to play a bigger role in cardiac development. It has been shown that p300 increases the DNA binding ability and stability of GATA-4, a key molecule in the cardiac lineage specification cascade (Kawamura et al., 2005); (Dai & Markham, 2001). Furthermore, in an *in vivo* study by (Yao, Oh, Fuchs, Zhou, Ch'ng, Newsome, Bronson, Li, Livingston, & Eckner, 1998b), the primary reason for homozygous p300 knockout mice lethality between embryonic day 9 to 11.5 was thought to result from the cardiac defects, despite these mice having normal levels of the CBP protein. In another study, homozygous CBP deficient mice died between embryonic days 10.5-12.5 due to hemorrhage and had a defective neural tube, but did not have the cardiac developmental defects seen in the CBP mutant Rubinstein-Taybi Syndrome humans and mice, this was in part thought to be due to the dominant negative effects of the mutant form of CBP as opposed to its deficiency (Tanaka et al., 2000). In contrast to these findings, CBP was found to be crucial differentiation into hematopoietic lineages (Kung et al., 2000).

Knock out studies of CBP and p300 in mice have also theorized that both p300 and CBP cause lethality in a dose dependent manner, as the least viability was seen in p300 and CBP heterozygote knockout combination mice, than CBP heterozygote or P300 heterozygote knockout alone. It was also seen that retinoic acid signaling was hindered in p300 knockout cells while CAMP signaling remained unaffected. (Yao, Oh, Fuchs, Zhou, Ch'ng, Newsome, Bronson, Li, Livingston, & Eckner, 1998a).

In addition to the functional differences observed between these proteins, they were also found to differ in terms of localization during embryonic development. A wide expression of p300 was observed in the cardiac crescent with CBP being absent on E 7.5, and CBP was more abundant in the heart tube on embryonic day 8.5 and at low levels in adulthood. Although the global expression profile among these two co-activators overlaps, p300 is thought to play a more important role in the induction of progenitor cells and the spatial organization of the developing heart (G. Chen, Zhu, Lv, Wu, Sun, Huang, & Tian, 2009a).

Role of P/CAF and GCN5 in Cardiac Development at a Cellular Level

Both CBP and p300 interact with the HATs P/CAF and GCN5. Both P/CAF and GCN5 are highly conserved complexes composed of 20 different TATA-box Associated Factor (TAF) polypeptides varying in size from 10-400kDa (Ogryzko et al., 1998). Gcn5p was first observed in yeast, with mouse and human GCN5 and P/CAF having additional 35 amino acids in comparison to the yeast HAT. This additional sequence confers the human and mouse GCN5 and P/CAF the ability to acetylate both free and nucleosomal histone substrates *in vitro.* P/CAF and GCN5 expression levels were found to be inversely related in most murine tissues (Xu, Edmondson, & Roth, 1998). A GCN5 and P/CAF knockout study showed that GCN5 null mice

embryos died by E10.5 following severe developmental retardation by E8.5. This was in part due to increased apoptosis impairing the formation of the mesoderm lineages that gave rise to somites and neural tube defects were also seen; while the extraembryonic and cardiac mesoderm development remained normal. Mice lacking both GCN5 and P/CAF showed more developmental abnormalities, indicating some similarity in functioning of these two HATs (Xu et al., 2000). On a similar note, a similar knockout study by Yamauchi and colleagues (2000) (Yamauchi et al., 2000) found differences in the viability among different mice. Mice lacking both P/CAF and GCN5 died and were absorbed during gestation before E9.5, mice lacking only GCN5 died between E9.5 and E11.5, with the P/CAF null mice developmentally similar to the wild-type and viable. These results indicate that the GCN5 may play a more crucial role during embryonic development than P/CAF. In another study that used GCN5 null ES cells, it was observed that GCN5 null ES cells were able to survive, form embryoid bodies, and differentiate into cells of the three germ layers including muscle lineages, *in vitro*, but died *in vivo*. Moreover, in the same study there was an early decrease in stemness markers Oct4 and Nodal in the GCN5 null ES cells, indicating the potential role of GCN5 in maintaining pluripotency (W. Lin et al., 2007).

This information taken together with the absence of abnormal muscle development in P/CAF null mice may indicate that both GCN5 and P/CAF are not essential for myogenesis. This is surprising, given that an earlier study indicated that P/CAF directly binds MyoD, acetylating it at conserved lysine residues 99, 102, and 104 causing a conformational change and increasing its DNA binding ability in *in vitro* and *in vivo* experiments; hence essential for skeletal myogenesis (Sartorelli et al., 1999). These controversies may indicate that P/CAF and GCN5 may not be essential for cardiomyogenesis, but P/CAF may be essential for skeletal muscle development.

However, more studies, including GCN5 and P/CAF knockout experiments focusing on muscle development from a molecular perspective are needed to clarify if P/CAF and GCN5 have distinct roles in progenitor cell specification, especially during myogenesis.

There is variability in the literature with regards to the compensatory expression seen in P/CAF and GCN5 null mice. P/CAF null mice did not have an increase in GCN5 at mRNA or protein levels, and were phenotypically normal (Xu et al., 2000). However, in another study, P/CAF null mice had an up regulation in GCN5 expression particularly in the lung and liver (tissues with high GCN5 expression in wild-type mice), but GCN5 levels in the brain remained low (organ with low level of GCN5 activity in wild-type mice) (Yamauchi et al., 2000). This indicates that GCN5 maybe compensating for P/CAF's role in these tissues. This discrepancy might perhaps be related to the slight differences in the null mice models generated, as well as the timing of the *in situ* hybridization and protein studies. Nevertheless, both these studies showed that mice lacking GCN5 died before E11.5, without comment on P/CAF levels in these mice. In Yamauchi and colleagues (2000) study, P/CAF transcription took place on E12.5 in wild-type mice, and GCN5 null mice embryos were dead before that time point was reached. There are also differences in the expression profile of P/CAF and GCN5. In an *in vivo* study P/CAF was not expressed until E12.5 and particularly abundant in the liver, heart, hind limbs and the skeletal muscle; while GCN5 had a more global expression pattern starting as early as E8 (Yamauchi et al., 2000). Nonetheless, more studies are needed to clarify if these HATs are able to functionally compensate for each other, and whether this compensation is tissue and developmental stage specific.

Effect of Transcriptional Co-activators on the Adult Heart

Aside from the role of HATs in normal embryonic development, HAT upregulation has also been associated with hypertrophy, similar to the consequences of fetal gene program activation during adulthood. For instance, ectopic overexpression of p300 and CBP stimulates hypertrophy in neonatal rat (Sprague-Dawley) cardiomyocytes (Gusterson, Jazrawi, Adcock, & Latchman, 2003), and transgene-mediated expression of p300 in the adult mouse heart also results in hypertrophy and heart failure (Yanazume et al., 2003). p300 acetylates proteins such as the zinc-finger GATA binding protein 4 (GATA-4), NK2 transcription factor related, locus 5 (Nkx2-5), MADS box protein Serum Response Factor (SRF), Rel transcription factor Nuclear factor of activated T-cells (NFAT), and the cAMP-responsive transcription factor CREB; thereby enhancing their activity, and facilitating the activation of the fetal gene program (Dai & Markham, 2001), (Kakita et al., 1999), (Slepak et al., 2001), and (Sartorelli, Huang, Hamamori, & Kedes, 1997). In addition to this, Muscle Enhancer Factor2 (MEF2) transcription factor is also activated and regulate the expression of many downstream structural proteins like β-Myosin Heavy Chain (β-MHC). MEF2 interacts directly with p300 and class II HDACs in a mutually exclusive manner on the same MEF2 domain (Sartorelli et al., 1997) and (Han, He, Wu, Liu, & Chen, 2005). Consequently, when class II HDACs are exported out of the nucleus in response to hypertrophic signals, MEF2 factors interact with p300 and become fully transcriptionally active, thereby activating downstream genes harboring the MEF2 binding sites.

These studies indicate that p300 and CBP play similar yet distinct time dependent roles in progenitor cell commitment, developmental morphogenesis, fetal and hypertrophic cardiac development.

Signaling During Cardiomyogenesis

The pathways involved in cardiomyogenesis are partly unclear, and those elucidated are highly complex. Many of the molecules involved in these cascades have different roles not only in cardiac lineage specification, but also in differentiation into other tissue types depending on the time point in development.

The inner mass cells in the blastocyst stage are pluripotent during embryonic development. In a study by Boyer and colleagues (2005), human embryonic stem cells were found to express Oct4 a member of the POU (PIT/OCT/UNC) class of homeodomain proteins, SOX2 an HMG-box transcription factor, and NANOG a homeobox transcription factor(Boyer et al., 2005). These three transcription factors are highly conserved among species and in this study were found to collectively bind 353 genes in the human embryonic stem cells. In 90% of the cases these 3 regulatory proteins were found bound close to each other on their target genes. These transcription factors were thought to regulate not only their own expression by binding pattern, but also the expression of members of the Tgf-β and wnt pathway (Boyer et al., 2005). Both these pathways have been implicated in the maintenance of pluripotency in murine and human stem cells ((James, Levine, Besser, & Hemmati-Brivanlou, 2005) and (Sato, Meijer, Skaltsounis, Greengard, & Brivanlou, 2004)). Wnt/β catenin was found to play a dual role during the differentiation of murine ES cells and zebrafish embryonic cardiac development. When ES cells were treated with Wnt-3A early on, the induction of the precardiac mesoderm (marked by Mesp1 and Brachyury T) was enhanced and created a negative feedback loop suppressing Wnt-3A and Wnt-1 while increasing the expression of Dkk1 (an inhibitor of the Wnt signaling pathway) thereby increasing cardiomyogenesis. However, once the cells were specified Wnt signaling inhibited cardiomyogenesis. The *in vivo* activation of Wnt signaling before gastrulation in

zebrafish was also more favorable for cardiomyogenesis (Ueno et al., 2007). In a more recent study on murine embryonic fibroblast cells focusing on the effects of SUMOlyation, it was found that the addition of small ubiquitin like SUMO modifiers to Oct4 and SOX2 impaired the formation of the heterodimer. This in turn prevented the binding of Oct4 and SOX2 to the NANOG regulatory elements and repressed its expression. However, the compartmentalization of Oct4 and SOX2 remained unaffected and nuclear. This study also found that individual SUMOlyation of Oct4 and SOX2 had opposite effects on NANOG, for instance SUMOylation of Oct4 increased NANOG expression while that of SOX2 decreased it shedding new insight into potential mechanisms for pluripotency maintenance and differentiation (Wu et al., 2012). Additionally, CBP/p300-interacting transactivator with glutamic acid (E)/aspartic acid (D)-rich tail 2 (Cited2) was found to play a role in pluripotency and differentiation. Cited2 knockout ES cells, continued to express the stemness genes Oct4, Klf4, Sox2, and c-Myc, and the impairment in Cited2 recruitment to the Oct4 promoter hindered differentiation into cardiomyocyte, hematopoietic, and neuronal lineages (Q. Li, Ramirez-Bergeron, Dunwoodie, & Yang, 2012).

NANOG is important for the maintenance of stemness (Mitsui et al., 2003). In a more recent study, it was found that decrease in NANOG was associated with differentiation, with p53 binding having a potential mechanistic role in this repression in murine ES cells through direct binding to the NANOG gene. In this study, it was also seen that retinoic acid treatment (induces ES differentiation) decreased NANOG levels, surprisingly the occupancy of p53 was reduced, the decrease in NANOG expression in this case was potentially related to the reduction in histone acetylation observed at its promoter (T. Lin et al., 2005). Moreover, Oct3/4, another member of the POU family of transcription factors marking an undifferentiated state, was found to affect pluripotency and commitment of stem cells in a dose dependent manner. In ES cells, a

50% increase in the level of Oct3/4 tipped the cells from pluripotency to mesoderm lineage commitment, while a 50% decrease from baseline was associated with the formation of the trophectoderm. The increase in Oct3/4 was not sufficient to maintain pluripotency in the absence of LIF (Leukemia Inhibitory Factor), and led to differentiation. This also correlated with the increase in GATA-4 and BrachuryT expression indicating mesodermal lineage specification, indicating the importance of Oct3/4 threshold during differentiation ((Niwa, Miyazaki, & Smith, 2000).

In vertebrates the cardiac cells originate from the anterior lateral mesoderm, with the anterior endoderm secreting factors to kick start the lineage specification in the nearby mesoderm (Sugi & Lough, 1994). These factors include members of the BMP, Shh, FgF, Wnt, and Notch signaling pathway ((Zaffran & Frasch, 2002). In particular, BMP4 was expressed in the mesoderm itself and BMP2 and BMP7 were expressed in the anterior lateral endoderm, with BMP factors needed to maintain Nkx2-5 and GATA-4 gene expression following the fusion of the bilateral heart fields but not for their initiation (Schultheiss, Xydas, & Lassar, 1995). However, in a more recent study it was found that the mouse mesoderm, including the normally noncardiogeneic posterior and extraembryonic mesoderm, could be induced to transdifferentiate into cardiac cells by GATA-4, TBX5, and Baf6oc gene expression (Takeuchi & Bruneau, 2009).

Notch and Wnt signaling also plays an important role during multipotent cardiogenic cell proliferation and differentiation. Notch signaling is triggered upon the activation of Notch receptors by their ligands Delta or Jagged through direct interaction among the cells. The Notch receptor is then cleaved following this activation, and the resulting NICD is nuclearized forming a complex with the transcription factor RBP-Jk ((Ehebauer, Hayward, & Martinez-Arias, 2006). On the other hand, following the activation of Wnt signaling by ligand binding the GSK3β

kinase, which normally marks β-catenin for degradation, is inhibited. This increases cytoplasmic β-catenin which is then translocated to the nucleus, regulating transcription through LEF and TCF ((Katoh & Katoh, 2007); (Nakamura, Sano, Songyang, & Schneider, 2003). Integrin-linked kinase (ILK) was found to phosphorylate GSK3β kinase and negatively regulate it; promoting the Wnt signaling cascade (White et al., 2006). In another study, *in vitro* overexpression of ILK was found to augment cardiac lineage commitment ((Traister et al., 2012). This was potentially thought to be due to the repression of Oct4 by Tcf3 (a mediator of β catenin activity (Tam et al., 2008).

ISL1, a homeodomain containing transcription factor, is a multipotent cardiac progenitor cell (CPC) marker expressed shortly before cell migration to the heart tube. ISL1 is directly regulated by β catenin and essential for the proliferation, migration, and viability of cardiac progenitor cells, such that its ablation results in death at E.13 (W. Lin et al., 2007). A murine *in vivo* study by Kwon and colleagues (2009) has found that Notch1 and β-catenin signaling pathways cross-talk. In this study, Wnt/ β catenin signaling facilitated CPC population. This was repressed by the Notch1 signaling that positively regulated the expression of cardiac transcription factors ISL1, myocd, and smyd1 by reducing the active β catenin; thereby promoting differentiation (Kwon et al., 2009). It was also shown that Notch1 played different roles depending on when it was activated during differentiation signaling, promoting cardiomyogenesis in the undifferentiated P19CL6 cells and inhibiting it on day 3 (B. Li et al., 2012; B. Li et al., 2012). Another study found similar temporal effects in Wnt signaling (Ueno et al., 2007). Moreover, the study by Li and colleagues (2012), found that molecules from the two cascades, RBP-Jk and NICD-1 complex to regulate the Hes1 gene (B. Li et al., 2012; B. Li et al., 2012); however, the authors did not comment on the implication of this finding on formation of the cardiac lineage.

Nevertheless, these findings underline the temporality of cardiomyogenesis as well as the interaction between these two pathways.

Additionally, the growth factor stimulated PI3K/Akt signaling pathway also seems to be involved in cardiomyogenesis depending on its intracellular localization. A study by Fischer and colleagues (2011) revealed that over expression of nuclear Akt in CPCs increased their proliferative capacity while impairing cardiac lineage commitment (Fischer et al., 2011). In contrast to this finding, it was shown that Akt signaling was positively involved in cardiac differentiation, serving as an upstream effector to the cardiac specific Cx 43 and Nkx2.5 expression. In this study, mechanical strain on ES cells was found to upregulate intracellular ROS, which was then thought to modulate integrin β1, thereby activating PI3K/Akt (Heo & Lee, 2011). The seemingly dual role of PI3K/Akt signaling on cardiomyogenesis may stem from a dose dependency, mode of cascade activation, or the involvement of other factors that have confounding effects and remain yet to be elucidated. More studies are therefore needed to clarify the role of PI3K/Akt signaling in cardiomyogenesis.

Ribosomal S6 Kinase (S6K) modulates gene expression at the mRNA translation level. It is regulated by the PI3K/Akt and mTOR signaling pathways independently (detailed review (Gingras, Raught, & Sonenberg, 2001)). A recent study found that S6K plays a role in the commitment and gene expression of ES cells. siRNA transfection deterred the formation of beating cardiomyocytes and the expression of the cardiac genes possessing 5' terminal oligopyrimidines Cx43, Dsp, and PTEN in ES cells. Moreover, S6K knockdown ES cells differentiated into neurons under cardiogenic conditions, thereby potentially playing as a gatekeeper to prevent neurogenesis (L. Li et al., 2012). However, the regulatory effect of this

ribosomal kinase, if any, on the early cardiac lineage commitment and differentiation markers like ISL1 and GATA-4 remains yet to be studied.

The initiation of cardiac differentiation is indicated by the expression of the cardiac early marker genes, which include GATA binding protein 4 (GATA-4), myocyte enhancer factor 2C (Mef2c), NK2 transcription factor related, locus 5 (Nkx2.5), and T-box 5 (Tbx5) (Uchida, Fuke, & Tsukahara, 2007). Wnt-3a and Wnt-8a factors are also thought to play a role in the early initiation of differentiation during embryonic development (Ohara, Atarashi, Ishibashi, Ohashi-Kobayashi, & Maeda, 2006). In a more recent study, it was shown that Wnt-3a increased the phosphorylation of Akt and GSK3β, and augmented β-catenin accumulation in the nucleus in a throphoblast motility study (Sonderegger et al., 2010). This finding provides a potential convergence point for these pathways, however, studies are needed to determine the validity of this finding during cardiomyogenesis. The complex interactions between these various cascades is summarized in Figure 2.

The GATA transcription factor family is an evolutionarily conserved zinc finger containing protein family which recognizes the WGATAR consensus DNA sequence ((Molkentin, 2000); (Patient & McGhee, 2002)). It consists of six members in mammals, of which GATA-1, -2, -3 regulate hematopoiesis and nervous system development, and GATA-4,-5,-6 are important for heart and gut development and are 85% identical within the zinc finger and basic regions (Molkentin, 2000). Slight variation in the DNA core motif of GATA members is thought to account for variance in binding when co-expressed in any given tissue at a given time (Molkentin, 2000). GATA-4 (48kDa) is a key regulator of endoderm and mesoderm development in the post gastrula embryo ((Heikinheimo, Scandrett, & Wilson, 1994); (Rossi, Dunn, Hogan, & Zaret, 2001)). Studies have shown that GATA-4 knockout mice die around

9.5dpc due to defects in heart and gut morphogenesis and have an increase in the expression of GATA-6 ((Kuo et al., 1997); (Molkentin, Lin, Duncan, & Olson, 1997)(Molkentin, 2000)). GATA-4 also controls the expression of genes critical for cardiomyogenesis and regulates its own expression as well (Durocher, Charron, Warren, Schwartz, & Nemer, 1997). Moreover, its over expression in P19 embryonic carcinoma (EC) cells and mouse Embryonic stem (ES) cells is associated with increased cardiac differentiation (Grepin, Nemer, & Nemer, 1997).

The expression of GATA-4 is highly complex, with alternate enhancers and transcription initiation sites that display tissue and temporal specificity. Its role at any given time during embryonic development is also dependent on interactions with other factors co-expressed in the tissue, as mentioned earlier. An enhancer region named CR2 (-41 to -42kb) was determined to be essential for GATA-4 expression in the early mesoderm of mice in an in vivo study by Rojas and colleagues (Rojas et al., 2005). In an *in vivo* study by Heicklen-Klein and Evans (2004) it was shown that 1.1kb of the mouse GATA-4 promoter was essential for its expression, while the enhancer activity was varied depending on the timing and location of GATA-4 expression (Heicklen-Klein & Evans, 2004). The TATA-less promoter of the GATA-4 gene is highly conserved among human, mouse, and rat. However, the transcription start site is 30bp downstream in stomach tissue compared to cardiac and testis (Ohara et al., 2006). A study by Tiwari et al. revealed that the GATA-4 gene was in an unmethylated poised state with a rapid increase in transcription following stimulation with retinoic acid (Tiwari et al., 2008).

Additionally, GATA-4 interacts with many transcription factors to regulate differentiation into cardiomyocytes. Mef2c is a cofactor for GATA-4 and plays a role in differentiation to cardiac, skeletal, and smooth muscle (Morin, Charron, Robitaille, & Nemer, 2000). GATA-4 regulates the expression of the homeodomain containing transcription factor Nkx2.5 by

activation of its enhancer in combination with smads (Akazawa & Komuro, 2005). In addition to this, GATA-4 binds to Nkx2.5 causing a conformational change, thereby allowing it to bind to the promoter of the atrial natriuretic factor (ANF) which promotes cardiomyogenesis (Durocher et al., 1997). The transcription factor Nkx2.5 also associates with Tbx5 (a transcription factor involved in heart tube and chamber formation) (Stennard & Harvey, 2005) and binds to the natriuretic peptide precursor type A (Nppa) further promoting the generation of heart cells (Hiroi et al., 2001).

Hence, it seems that these early cardiac marker gene products specify, integrate, and amplify signals from different pathways for cardiomyogenesis by interacting and regulating each other.

However, the temporal expression of this early cardiac gene network appears to be key; as the activation of this fetal gene program in adulthood leads to hypertrophy, and the associated increase in pathologic sarcomeric and structural proteins (Sadoshima & Izumo, 1997).

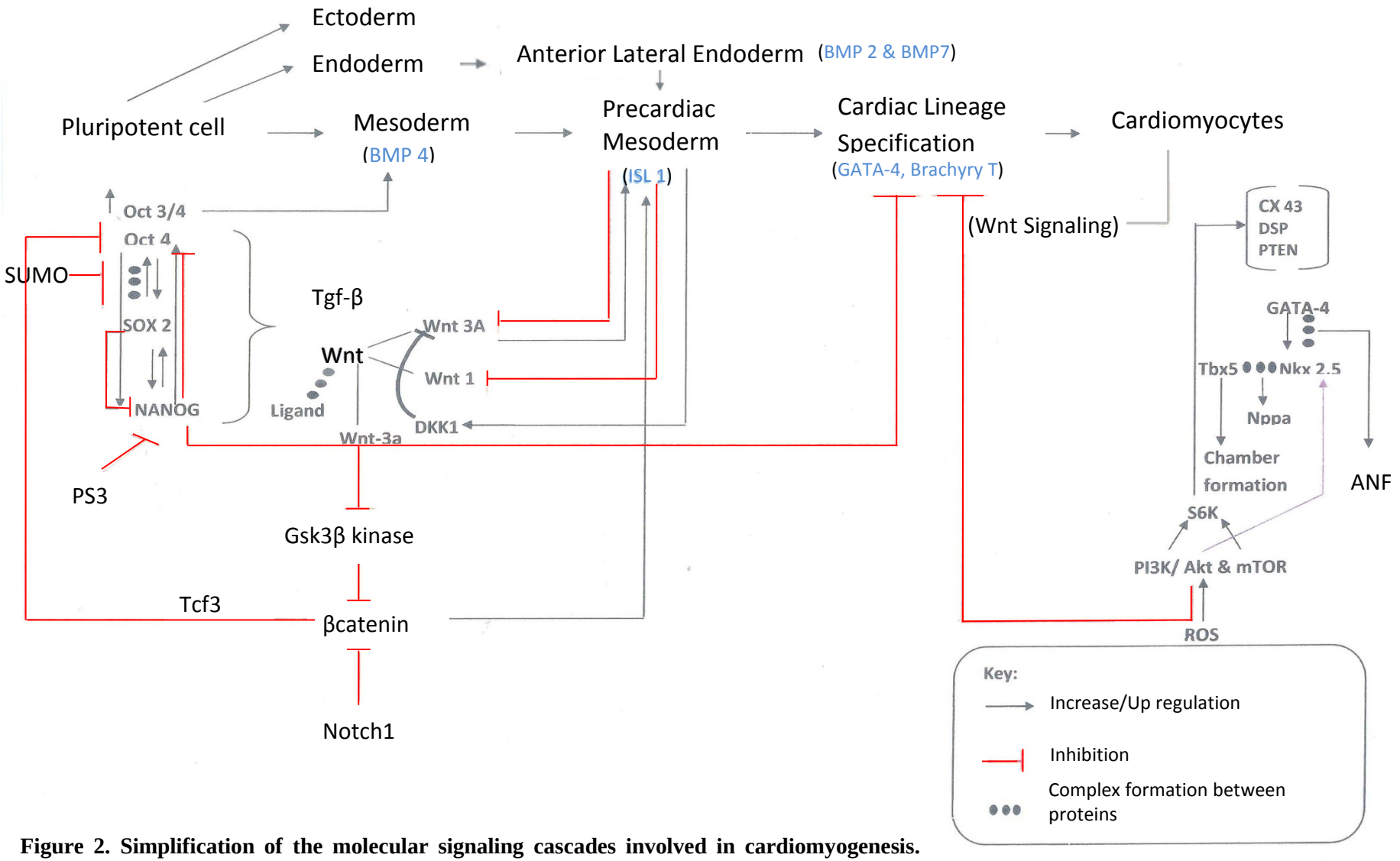

Figure 2. Simplification of the molecular signaling cascades involved in cardiomyogenesis. Schematic representation of the convergence of the NANOG, Wnt, Notch 1 signaling cascades, their associated factors, and roles during cardiac lineage specification and differentiation. Note that the Wnt signaling pathway could both up regulate and down regulate cardiomyogenesis depending on various factor levels.

Embryonic Carcinoma and Embryonic Stem Cell Lines to Investigate Cardiomyogenesis

The P19 EC cell line was derived from the primary tumor of a teratocarcinoma induced by transplanting a female embryo (E 8.5) from a cross between a C3H/He female and feral male into the testes of a mouse from a different strain. This cell line was isolated from the primary tumor, and was found to be pluripotent, capable of differentiating into cells from the three germ layers (endoderm, mesoderm, and ectoderm). Due to their ability to be maintained indefinitely, easy induction, high yield, and stable genetic manipulation with transfection makes it an ideal model to investigate differentiation ((McBurney, Jones-Villeneuve, Edwards, & Anderson, 1982); (McBurney, 1993)). P19 cells could differentiate into neurons and glial cells upon induction with 0.1-1uM Retinoic Acid (RA) when grown in a monolayer (McBurney et al., 1982). Skeletal and cardiac differentiation occurs upon aggregation and treatment with 0.1-1nM RA or 0.5-1% Dimethylsulphoxide (DMSO) and specific media ((McBurney et al., 1982); (Edwards, Harris, & McBurney, 1983); (Campione-Piccardo, Sun, Craig, & McBurney, 1985)). Mouse ES cells are derived from the inner cell mass of a blastocyst (3.5 days post coitus) ((Kaufman, Robertson, Handyside, & Evans, 1983); (Martin, 1981)). Like the EC cells they are pluripotent; however, they can differentiate into cells from all three germ layers spontaneously depending on the type of growth medium used (Doetschman, Eistetter, Katz, Schmidt, & Kemler, 1985). This model is attractive for use in differentiation studies, as these cells are easy to maintain, have high yield, and can be genetically modified ((Boyer, Mathur, & Jaenisch, 2006); (Niwa, 2007)). In addition to this, ES cells are closer in nature to human stem cells due to their embryonic origin as opposed to the cancerous P19 cells.

D3 Embryonic Stem cells (ES) are derived from the inner cell mass of embryos in the blastocyst stage (Evans & Kaufman, 1981). These cells are maintained in the undifferentiated state by the addition of Leukemia Inhibitory Factor (LIF), in contrast to the EC cells. The

LIF/Stat3 signaling is triggered by the binding of a cytokine to the LIF-R receptor leading to its dimerization with the gp130 receptor. This receptor complex then activates the JAK family of non-receptor tyrosine kinases leading to phosphorylation of the receptor dimers, followed by Stat3 recruitment and phosphorylation. This LIF/Stat3 pathway is then thought to converge with the parallel Wnt pathway (discussed earlier) on the expression of Myc, a helix-loop-helix/leucine zipper transcription factor whose expression has the capacity to maintain pluripotency even in the absence of LIF (Cartwright et al., 2005). Interestingly, LIF activated Stat3 was found to bind Tbx5, Nkx2-5, and GATA4 promoters in P19CL6 cells promoting cardiomyogenesis; while its RNAi knockdown reduced the number of beating cardiomyocytes (Snyder, Huang, & Zhang, 2010). ES cells are capable of differentiating into cells from all three germ layers after the formation of embryoid bodies (EB) in hanging drops in the absence of an inducer for differentiation, and the cardiomyocytes form between the external epithelial layer and a mesenchymal layer. Beating cardiomyocytes are observed 1-4 days after plating of the EB, and were found to be composed of cells similar to the early heart tube and myocardium; once fully differentiated cardiac cells contraction ceased (Boheler et al., 2002). In a study by Kolossov and colleagues (2005), the heart cells were seen to be a mixture of atrial, ventricular, and pacemaker(Kolossov et al., 2005).

Chemical Inducers in Cardiomyogenesis

Dimethylsulfoxide (DMSO) induces myogenesis in P19 cells when aggregated in 0.5%-1% concentration (Skerjanc, 1999). Its exact mechanism of action is largely unknown; however, it is thought to induce cardiomyogenesis by triggering the release of intracellular calcium stores without phosphoinositide breakdown (Morley & Whitfield, 1993). Studies have also indicated the importance of the Cx43 gap junctions and the oxytocin receptor system in mediating

DMSO's effects (Jasmin, Spray, Campos de Carvalho, & Mendez-Otero, 2010). Additionally, it was shown that DMSO increased the DNA methyltransferase Dnmt3a transcript levels in a dose dependent manner, and resulted in hypomethylation or hypermethylation in a locus specific manner (Iwatani et al., 2006). These findings indicate that DMSO can mediate differentiation and gene expression by various mechanisms.

Valproic acid (VPA) is fatty acid drug used to treat epilepsy, migraine pain, seizures, and bipolar disorder ((Johannessen & Johannessen, 2003); (Loscher, 1999)). Its exact mechanism of action is also unknown; however, studies have shown that VPA inhibits the activity of class I HDACs and induces the degradation of class II HDACs along with p300 thereby affecting gene expression ((Kramer et al., 2003); (Phiel et al., 2001)). From a mechanistic point, these changes suggest an increase the acetylation of histones, and could potentially induce cardiomyogenesis by facilitating gene expression. However, a study by Na and colleagues (2003) on murine ES cells found otherwise. In this study, VPA slowed EB development and decreased the formation of contractile cardiomyocytes in a dose dependent manner without cytotoxic effects; however, there was an increase in intracellular ROS levels. The increased ROS was found to be the cause of this inhibition (Na, Wartenberg, Nau, Hescheler, & Sauer, 2003).

Regardless of the inducer used, differentiation of EC and ES cells is also affected by factors found in the Fetal Bovine Serum (FBS) used for culturing these cells. These factors are not completely identified, and show variability among different batches of preparations (Wilton & Skerjanc, 1999). Therefore, in addition to determining the ideal concentration of the inducer for differentiation, it is equally important to test batches of FBS for finding the right match and maximize the efficiency of the intended lineage.

Curcumin, a selective p300/CBP HAT inhibitor

Curcumin (diferuloylmethane) is the main curcumanoid in the spice turmeric giving it a yellow color. It readily penetrates cells, has low toxicity and bioavailability when taken orally (Hsu & Cheng, 2007). Studies have shown that Curcumin has anti-tumor, anti-oxidant, anti-inflammatory effects ((Balasubramanyam, Varier, Altaf, Swaminathan, Siddappa, Ranga, & Kundu, 2004a); (Y. Chen et al., 2007), Marcu et al., 2006). Curcumin was found to suppress the activity transcription of Stat3 by inhibiting its phosphorylation contributing to its anti-cancer effects (Bharti, Donato, & Aggarwal, 2003). Moreover, Curcumin also inhibits the Wnt/β catenin pathway by increasing the degradation of β catenin by Caspase 3 distinctly from that resulting by GSK3β phosphorylation ((Jaiswal, Marlow, Gupta, & Narayan, 2002); (Park et al., 2005)).

Curcumin specifically inhibits HAT activity of p300/CBP, but the HAT activity of PCAF and methyltransferase and HDACs are also unaffected, both in vivo and in vitro (Balasubramanyam, Varier, Altaf, Swaminathan, Siddappa, Ranga, & Kundu, 2004a). Another study had similar findings, where the activity of HDACs where not effected in vitro. Additionally in this study it was observed that the HAT inhibition could not be counteracted with the administration of TSA (a HDAC inhibitor like VPA). This study also confirmed a dual role in the effect of Curcumin on ROS generation in a dose dependent manner, with higher concentrations of Curcumin increasing intracellular ROS levels, while lower levels decreased ROS levels (Kang, Chen, Shi, Jia, & Zhang, 2005). Curcumin is thought to mediate its effect by binding to a non-active site region of p300/CBP and causing a conformational change, thereby decreasing its efficiency to bind acetyl CoA (uncompetitive inhibition) and histones (competitive inhibition) (Balasubramanyam, Varier, Altaf, Swaminathan, Siddappa, Ranga, & Kundu, 2004a).This process takes place by a Micheal reaction, with the α and β unsaturated carbonyl side chains of Curcumin serving as acceptors with the formation of a covalent bond between p300 and Curcumin (Marcu et al., 2006).

Previous studies have shown the importance of p300 acetyltransferase activity, histone acetylation, and GATA-4 expression in cardiomyogenesis as well as in the activation of the fetal gene program post myocardial injury. However, the role of p300 on GATA-4 expression is yet to be elucidated. Our hypothesis is that the histone acetyltransferase activity of p300 may be involved in the gene expression of GATA-4. Therefore, the aim of this study is to investigate the effect of p300 histone acetyltransferase activity on GATA-4 gene expression at the molecular level during cardiomyogenesis using EC and ES cells, and the HAT inhibitor Curcumin.

METHODOLOGY

Cell culture and differentiation

P19 EC cells

P19 cells (ATCC CRL-1825) were maintained in the undifferentiated stage as monolayer in 75 cm^2 tissue culture flasks (Corning, 430720) in Minimum Essential Medium α (α-MEM) (Gibco-Invitrogen, 12000-022) supplemented with with 5% fetal bovine serum (Multicell Wisent), 5% donor bovine serum (Multicell Wisent), 1% Penicillin/Streptomycin (Gibco-Invitrogen, 15140122). The cells were incubated in Ster-Cycle Thermo incubator (Fisher Scientific) at 37°C with 5% CO_2.

Differentiation was initiated on Day 0, by aggregating the cells in 150mm Petri dishes (VWR, 25384-326) and treatment with 0.8 % DMSO (Sigma-Aldrich, D2650) or VPA 0.5mM (Sigma-Aldrich, P4543). The Embryoid Bodies (EB) were plated on tissue culture dishes (Corning, NY14831) for 3 more days, and on 0.1% gelatin coated coverslips until Day 6 or Day 8. Pellets were collected for western blot analysis on Day 4 and Day 7.

The HAT activity of CBP and p300 was inhibited by using10μM Curcumin (dissolved in 70% Ethanol) (Sigma, C7727-500MG), a concentration that was found to effectively inhibit with minimal cell death by previous experiments in our laboratory (results not shown). P19 cells induced with 0.8% DMSO were treated with Curcumin either between Day 0-2 or Day 2-4 of differentiation.

D3 ES cells

D3 cells (ATCC CRL-1934) were maintained in the undifferentiated stage as a monolayer in 75 cm^2 tissue culture flasks (Corning) in Dulbecco's Modified Eagle Medium (D-MEM) (Gibco-Invitrogen, 12800-017) supplemented with with 15% fetal bovine serum (Multicell Wisent), non-essential amino acids (Gibco, 11140), Beta-mercapthoethanol, 1% Penicillin/Streptomycin (Gibco-Invitrogen). Leukemia Inhibitory Factor (LIF) (Chemicon, ESG1107) at 1000U/ml of medium was added to maintain the pluripotency.

ES cells were aggregated in hanging drops for 2 days with the removal of LIF and aggregated for 6 additional days. EBs were plated on tissue culture dishes (Corning, NY14831) and on 0.1% gelatin coated coverslips (until Day 13). Pellets for western blots were collected on Day 6 and on Day 8.

The HAT activity of CBP and p300 was inhibited by using10μM Curcumin (dissolved in 70% Ethanol) (Sigma, C7727-500MG), a concentration that was determined by previous experiments in our laboratory (results not shown) to inhibit cardiomyogenesis without causing morphological abnormalities in the EB. Cells were treated with Curcumin between Day 0-2; Day 2-4; Day 4-6.

Immunofluorescence

P19 and ES cells were differentiated for the duration indicated above. After attaching to the cover slips, the cells were fixed with ice cold 100% methanol. The cells were air dried at room temperature followed by rehydration in 1xPBS, and left in primary antibody diluted in 1xPBS overnight at 4ºC overnight. The cells were incubated in secondary antibody diluted in 1xPBS for 2 hours at room temperature (21^{o} C), and washed with 1xPBS three times before nuclear staining

with 0.1µg/ml Hoechst (Molecular Probes) for 5minutes and washed with 1xPBS three more times before setting it on the slide with 10% glycerol.

Following staining, the coverslips were visualized using Axiovert 200M microscope (Zeiss), AxioCam HRM camera (Zeiss) and AxioVision Rel 4.6 software (Zeiss). The percentage of differentiation was estimated based on the fraction of the differentiated cells (based on the staining). The primary antibodies used were anti-MHC (1:10 dilution, homemade from MF 20 hybridoma cell line), anti-cTnT (1:200, Abcam, ab8295-200). The secondary antibodies used were Alexa Flor®488 goat anti-mouse (1:200, Invitrogen, A11001). Student *t*-tests were used for statistical analysis.

Western blotting

Cell pellets were collected during the differentiation as mentioned above and washed with 1xPBS. The cells were lysed in whole cell extract buffer (10 % glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 % NP-40) for 30 minutes on ice. Bradford assay was conducted using a Bio-Rad Protein Assay Dye Reagent (Bio-Rad, 500-0006) and Multiscan Spectrum photospectrometer (Thermo) to quantify the protein. Equal amounts of protein from each condition was diluted in 2X Laemmli buffer (25 % glycerol, 125 mM Tris-HCl pH 6.8, 4 % SDS, 10% β- mercaptoethanol, 0.01% bromophenol blue). Samples were heated to denature the proteins for 5 minutes at 95°C, and then separated on 6% SDS-polyacrylamide gel at 100V with Precision Plus Protein Kaleidoscope ladder (Bio-Rad, 10022141 RevA). The proteins were then transferred overnight onto Immun-Blot PVDF membrane (Bio-Rad, 1620177) at 35V, and the PVDF membrane was blocked for 1 hour with 5% non-fat milk in PBST (1% Tween in 1xPBS). The membranes were left in the respective primary antibody at 4°C overnight diluted with 1% milk in PBST. The next day, the membranes

were washed three times with PBST and incubated with secondary antibody for 1 hour at room temperature, and then washed again three times in PBST. The proteins were visualized using Western Lightning™ Chemiluminescence (Perkin Elmer, Nel105) reagents. The bands were quantified using Scion Image software (Scion Corporation). Membranes were re-probed after a 30 minute incubation in stripping buffer (2 % SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol) at 50°C, and blocked again with 5% milk in PBST for 1 hour. The membranes were then re-probed with another primary antibody as described above.

The primary antibodies used were: anti-p300 (Santa Cruz, N-15, sc-584) in 1:500 dilution, anti-CBP 1:500 dilution (Santa Cruz, C-20, sc-583), anti-GATA4 1:500 dilution (Santa Cruz, H-112, sc-9053x), anti-β-tubulin (homemade from E7 hybridoma cell line) in 1:100 dilution. Both MF20 and E7 cell lines were a gift from Dr. Alexandere Blais.

Chromatin Immunoprecipitation

P19 EBs were differentiated as described above, and crosslinked with 1% formaldehyde for 15 minutes at 37°C on Day 4 after the aggregates were broken up by repeated manual pipetting for 5 minutes. The formaldehyde was quenched with 200mM glycine. The cells were washed twice with ice-cold 1xPBS, and then lysed using ChIP Lysis Buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 1%SDS, 1X protease inhibitors (Roche, 03115879001), 1mM DTT, 1mM PMSF, 20mM NaButyrate) for 10 minutes on ice. The lysate was sonicated for 30 minutes with the Bioruptor system (Diagenode), using a 30s on/ 40s off cycle at high setting, and pelleted by centrifugation at 14,000rpm for 20 minutes. The supernatants were transferred into clean cold 1.7ml tubes.

DNA was quantified by taking aliquots from the conditions that were reverse-crosslinked, treated with DNAase free RNAse A (Sigma, R-4875), and ProteinaseK (Roche, 03115879001),

and purified for quantification analysis with NanoDrop spectrophotometer (ND-1000). The desired amount of chromatin was taken from the samples, diluted in ChIP Dilution Buffer (20mM Tris-HCl pH8.0, 150mM NaCl, 2mM EDTA, 1% Triton X-100,1x Protease inhibitors, and pre-cleared by incubation with TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) washed Dynabeads Protein A (Invitrogen, 100.02D) for 1 hour at 4°C.

After DNA quantification, the same amount of chromatin (10-15 μg) was taken from each crosslinked condition, and the volume was adjusted with ChIP dilution buffer. Samples were incubated with 2μg anti-p300 (Santa Cruz, N-15, sc-584), anti-CBP (Santa Cruz, C-20, sc-583). As well as, (2.5-5 μg) of chromatin was incubated with anti-H3KAc (Millipore,06-599). Anti-normal IgG was also used as a negative control according to the amount of chromatin added for the respective antibody animal source. All samples were rotated on VWR tube rotator overnight at 4°C.

The chromatin-antibody complexes were pulled down by 2 hour incubation with Dynabeads Protein A (Invitrogen, 100.02D). The beads were then washed with Washing Buffer A (20mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 150mM NaCl), Washing Buffer B (20mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 500mM NaCl), Washing Buffer C (20mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 1% NP-40, 1% sodium dioxycholate, 0.25 M LiCl) for 15 minutes per wash at 4°C, The samples were washed twice with TE, each wash being 5 minutes at 4°C. The chromatin was eluted from the beads with Elution Buffer (T_{50},$E_{10}S_1$ Tris 50mM, EDTA 10mM, SDS 1%) by rotating at room temperature for 30 minutes. The eluted samples reverse- crosslinked by incubation overnight at 65°C.

Samples were treated with DNAase free RNAseA (Sigma, R-4875), and ProteinaseK (Roche, 03115879001) the following day. All DNA purification was performed using Cycle Pure Kit

(Omega, D6492-02). The sequences were quantified by Real time QPCR (Stratagene Mx3000P, Agilent Technologies) using equal amounts of elutes. The primers are listed in Table 1.

The $2^{-\Delta\Delta Ct}$ value was calculated, where $\Delta Ct = Ct_{condition} - Ct_{condition\ input}$, and $\Delta\Delta Ct = \Delta Ct_{condition} - \Delta Ct_{\ undifferentiated\ reference}$. The fold increase was determined with respect to the undifferentiated control, with input chromatin DNA used as an internal control.

Table 1. Sequences of the ChIP primers used.

Target	Forward	Reverse
GATA-4 promoter*	5'-AAG CGC TCT TTT CTC CTT CC-3'	5'-GTG AGG GCT ACA GGG AGT GA-3'
GATA-4 control**	5'- GAG CTG GTA CCT GGC CTT C-3'	5'-GCT CTG CTG AAA TCA CTC TGA-3'

*GATA-4 primers were obtained from Voronova et al., 2012.
** GATA-4 control region primers were designed to target the GATA-4 CR2 enhancer region specified in Rojas et al., 2005.

RESULTS

Previous studies have shown that treatment with 0.5-1% DMSO induced P19 cells to differentiate into skeletal and cardiac myocytes (Skerjanc, 1999); with 0.8% being commonly used to study cardiomyogenesis ((Wilton & Skerjanc, 1999); (Gianakopoulos & Skerjanc, 2009)). It has also been known that HDAC inhibitors play a role in inducing differentiation of pluripotent cells into different lineages (reviewed in (Kretsovali, Hadjimichael, & Charmpilas, 2012)). Given these findings we decided to compare the efficiency of cardiomyogenesis with DMSO and VPA treatment, a more physiologic molecule that is widely used therapeutically as mentioned earlier. Prior experiments conducted in our laboratory have demonstrated a dose dependent effect of VPA on cardiomyogenesis in P19 EC cells, with a concentration of 0.5mM VPA being effective and least toxic (results not shown).

Small Molecule Inducers of Cardiomyogenesis

P19 EC cells were treated with either 0.8% DMSO or 0.5mM VPA and aggregated for 4 days before harvesting for western blotting and plating for staining on Day 6 Myosin Heavy Chain (MHC), and Day 8 Cardiac Troponin T (cTnT). The percentage of cardiomyocytes observed with DMSO treatment (12.14%) was almost twice as much as that observed with VPA treatment (6.93%) with MHC staining (Figure 3A and B). These findings were similar with cTnT staining (DMSO 10.95% and VPA 4.03%), with an overall higher percentage of differentiated cells seen with MHC staining than cTnT staining for both treatment types (Figure 3A and B).

Cell pellets were collected on Day 4 and Day 7 for both treatments, and GATA-4 expression was investigated. Globally speaking, GATA-4 expression increased from Day 4 to Day 7 with both modes of induction, being more pronounced with the VPA treatment (Figure 3C).

Surprisingly, in contrast to the microscopy results; treatment with VPA resulted in relatively higher expression of GATA-4 protein than with DMSO treatment on Day 4 and Day 7.

P19 EC cells were aggregated with DMSO and cross-linked with 1% paraformaldehyde and ChIPed with p300. Treatment with 0.8% DMSO also increased p300 occupancy at the GATA-4 promoter, with a fold induction of 16 in comparison to the undifferentiated control. The occupancy of p300 was higher following 0.5mM VPA treatment, with a fold induction of about 27 (Figure 3D).

Nevertheless, given the higher efficiency of cardiomyocyte generation observed with DMSO treatment, in comparison to VPA, DMSO was the inducer chosen to study the AT activity of p300/CBP in further experiments.

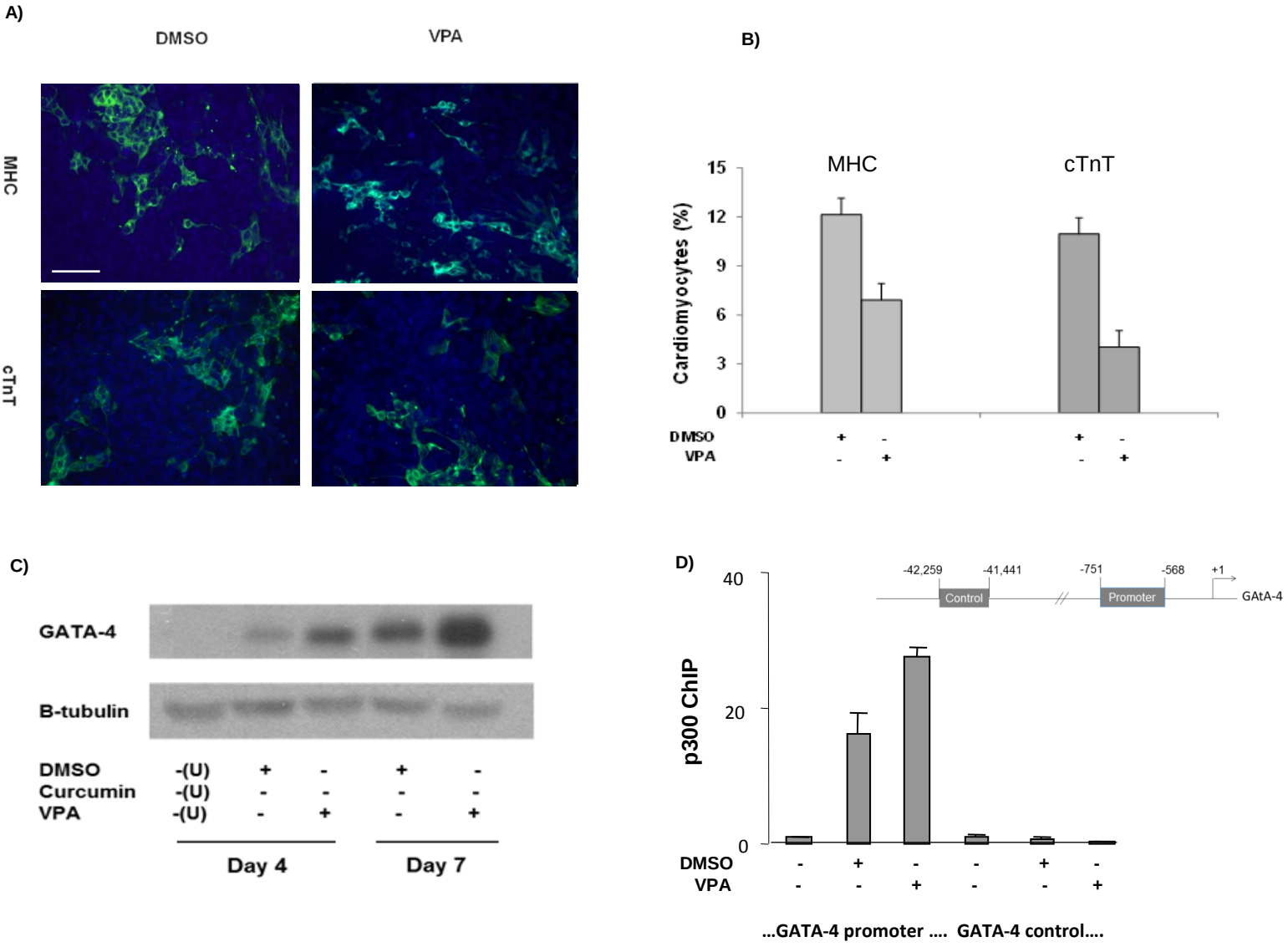

Figure 3. DMSO is a better inducer of cardiomyogenesis than the HDAC inhibitor VPA.
P19 cells were aggregated for 4 days in the presence of DMSO (0.8%) or VPA (0.5mM) until Day 4. A) Cells were stained with Myosin Heavy Chain (MHC) (green) on Day 6 and Cardiac Troponin-T (cTnT) (green) on Day 8, nuclei (blue) stained with Hoschest (Scale bar = 50µm). (B) Percentage of cardiomyocytes observed with MHC and cTnT staining following treatment. (C) Western blot for EC cell samples collected on Day 4 and on Day 7. Cell extracts were probed for GATA-4 with β-tubulin as the loading internal control. –(U) represents undifferentiated EC cells. D) EC cells were aggregated for 4 days and cross-linked with 1% PFA, the occupancy of p300 at the GATA-4 promoter (-568 to -751) of EC cells on Day 4 was analyzed, with primers designed for non-promoter (CR2) region of GATA-4 used as a control (-41.441kb to -42.259kb).

Early HAT Inhibition during Differentiation Impairs Cardiomyogenesis in EC cells

In this study, 10μM of Curcumin was used to inhibit histone acetyltransferase activity in the P19 EC cells. Previous studies have demonstrated that this concentration of Curcumin inhibits 50% of p300 HAT activity and is the least toxic concentration to cells (Francetic et al., 2012). In P19 EC cells, over eight fold reduction in the percentage of cardiomyocytes (Myosin Heavy Chain staining (MHC)) was observed with early Curcumin (10μM) treatment (Day 0-2) 1.25%, compared to DMSO treatment alone (10.5%) (Figure 4A and B). This reduction in the early treatment with Curcumin is even more pronounced with Cardiac Troponin T staining (cTnT), decreasing from 8.75% in the DMSO only treatment to 0.1% in early Day 0-2 treatment with Curcumin (Figure 4A and B). Treatment with Curcumin on Day 2 to 4, also decreased the percentage of cardiomyocytes observed (9% MHC and 7.05% cTnT). Similary, cTnT staining yielded a lower percentage than MHC for late Curcumin treatment (Figure 4B).

Inhibition of AT activity during Day 0-2 and Day 2-4 by 10μM Curcumin treatment in P19 EC resulted in a decrease in the GATA-4 protein levels compared to the uninhibited 0.8% DMSO treatment alone on Day 4. Interestingly, the decrease in GATA-4 protein was not regained by Day 7. This is in contrast to the increase in GATA-4 protein levels seen with the late Day 2-4, Curcumin inhibition treatment and DMSO treatment alone by Day7 (Figure 4 C and D). β-tubulin was used as the internal loading control in this experiment. Next, the role of AT activity was studied in the embryonic D3 ES cell line.

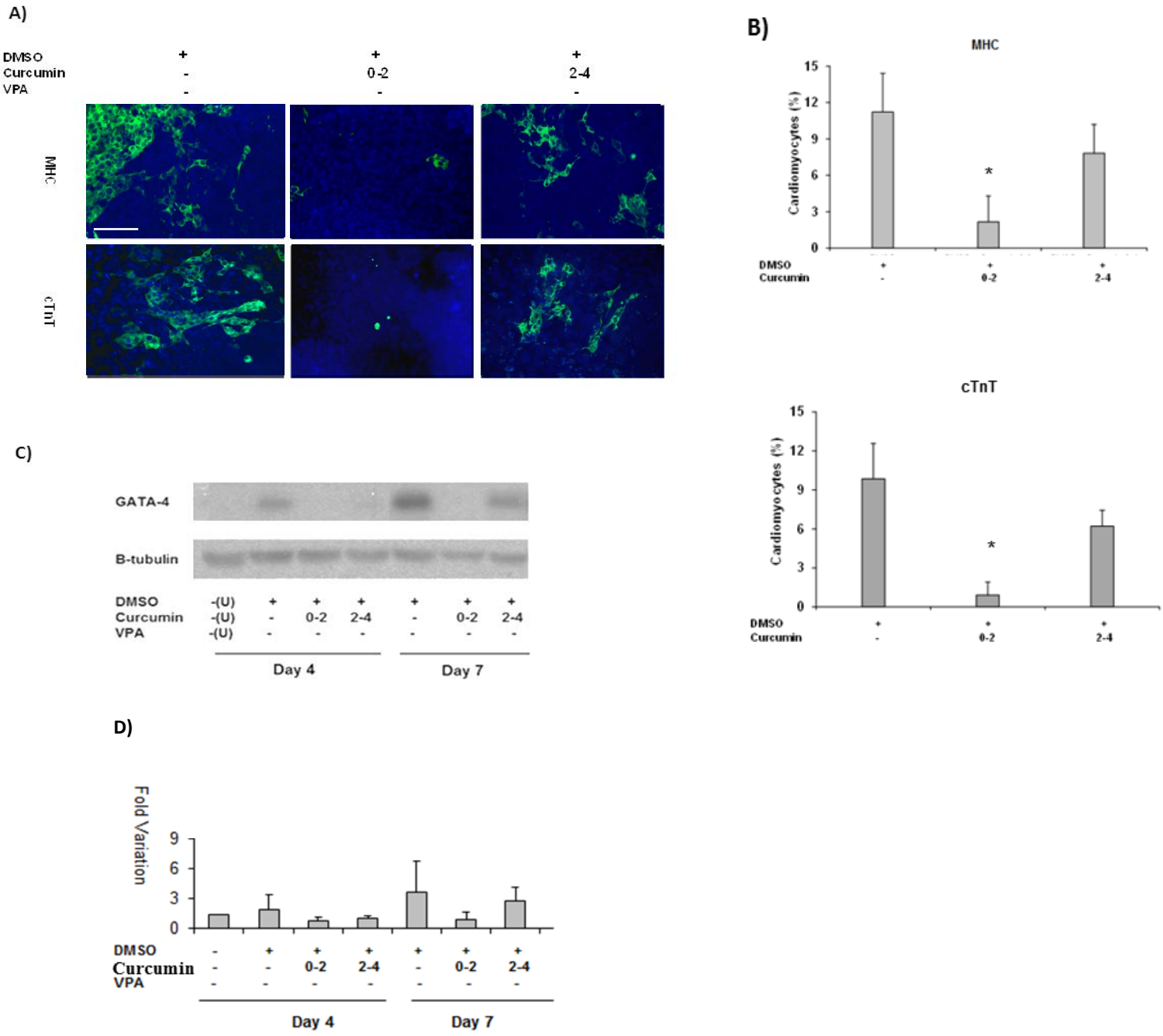

Figure 4. HAT activity is important during the early stage of cardiomyogenesis in EC cells.
(A) P19 cells were aggregated for 4 days in the presence or absence of DMSO (0.8%), Curcumin (10 μM) between Day 0-2 and Day 2-4 until Day 4. Cells were stained with Myosin Heavy Chain (MHC) (green) on Day 6 and Cardiac Troponin-T (cTnT) (green) Day 8, nuclei (blue) stained with Hoschest (Scale bar = 50μm). (B) Percentage of cardiomyocytes observed with MHC and cTnT staining following treatment. (mean ± SD, n=3) (C) Western blot for EC cell samples collected on Day 4 and on Day 7. Cell extracts were probed for GATA-4 with β-tubulin as the internal loading control. Samples were treated with DMSO (0.8%) and Curcumin (10μM) as indicated above. (D) Quantification of fold variation in western blot analysis.

Early HAT Inhibition during Differentiation Impairs Cardiomyogenesis in ES cells

ES cells were aggregated for 4 additional days following the 2 day hanging drop period and collected for western blotting on Day 6 and Day 8, and stained with MHC or cTnT on Day 13. A similar pattern of cardiomyogenesis inhibition emerged in ES cells with HAT inhibition using 10μM Curcumin inhibition as in the EC cells. HAT inhibition between Day0-2 caused the most reduction in cardiomyogenesis, with a 2.67 fold reduction with MHC staining and a 3.5 fold decrease seen with cTnT staining (Figure 5B) in comparison to no Curcumin treatment. The inhibitory effects of Curcumin on HAT activity affected cardiomyogenesis progressively to a lesser extent during treatments targeted at later time points of differentiation. The cardiomyogenesis observed with different treatments on MHC staining was 6.7% Day 0-2, 10.1% Day 2-4, and 15.77% Day 4-6 with the Day 4-6 treatment being closest to the uninhibited condition 17.9% (Figures 5A and B). Similarly, cardiomyogenesis was 3.73% Day 0-2, 7.87% Day 2-4, and 11.93% Day 4-6 with cTnT staining; and the Day 2-4 was closest to the uninhibited condition (13.3%) (Figures 5A and B). Overall, like the EC cells, cardiomyogenesis was higher with MHC staining than cTnT. The effect of HAT inhibition on GATA-4 expression at the protein level was investigated with western blots. The expression of GATA-4 in ES cells was decreased to a greater extent the earlier the Curcumin treatment on Day 6, with the highest decreased observed following Day 0-2 treatment. Although the level of GATA-4 expressed in the cells increased on Day 8 for all conditions, the level of expression remained considerably lower for the Day 0-2 treatment even on Day 8 (Figure 5C). This is in keeping with the inhibition of GATA-4 expression seen with the early Curcumin treatment. However, the extent of inhibition following the Day0-2 treatment was more pronounced in EC cells (Figures 4C and D). β-tubulin was used as the internal loading control. These results indicate the importance of AT activity during the early lineage commitment period.

Therefore, Chromatin Immunoprecipitation (ChIP) experiments were then undertaken to further investigate the molecular basis of GATA-4 gene expression during this early stage of cardiomyogenesis.

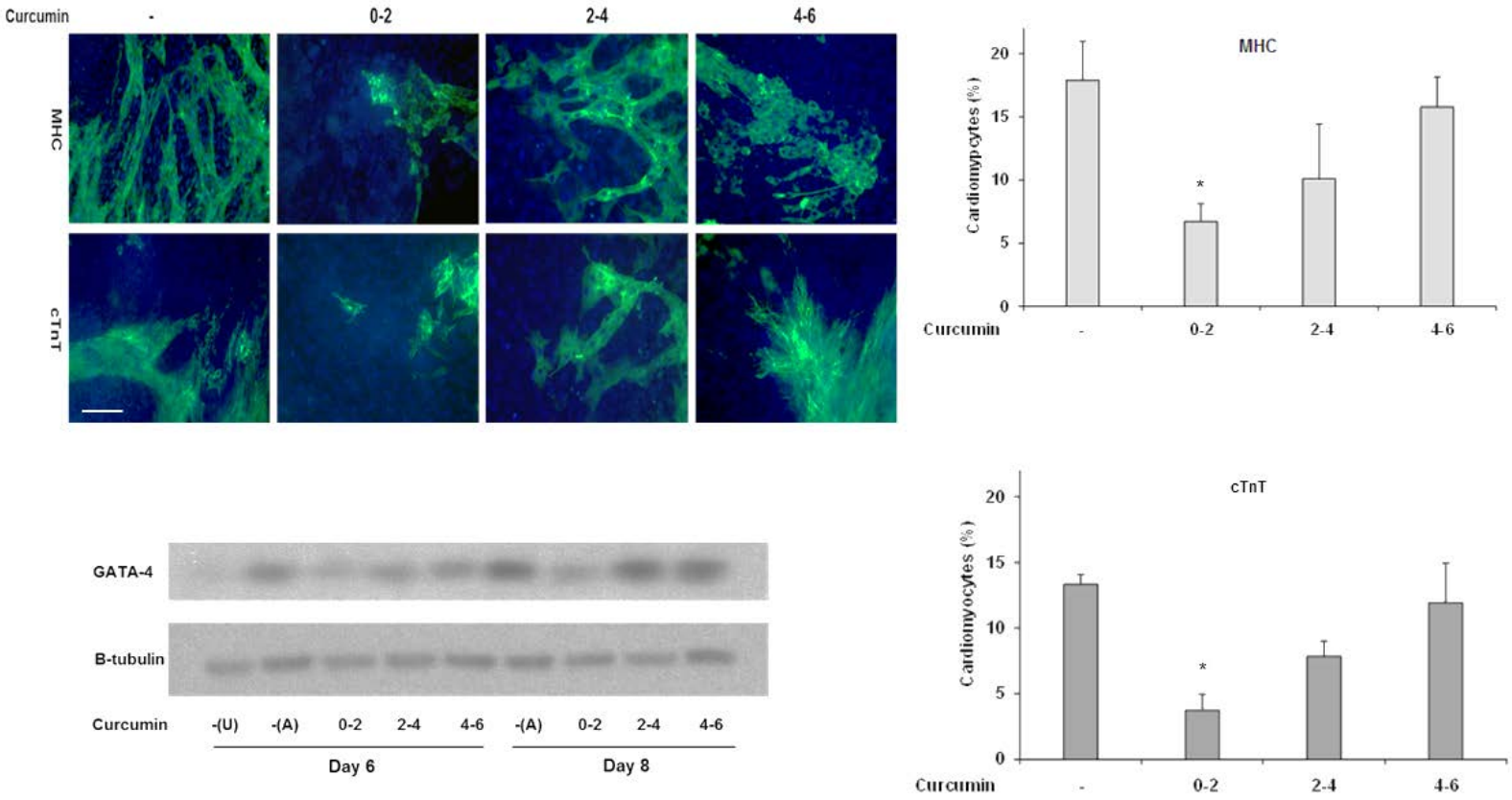

Figure 5. HAT activity is important during the early stage of cardiomyogenesis in ES cells. (A) ES cells were aggregated in hanging drops for 2 days and aggregated for 6 additional days and treated with Curcumin (10 μM) between Day 0-2; Day 2-4; Day 4-6 until Day 6. Cells were stained with Myosin Heavy Chain (MHC) (green) on Day 13 and Cardiac Troponin-T (cTnT) (green) Day 13, nuclei (blue) stained with Hoschest (Scale bars = 50μm). (B) Percentage of cardiomyocytes observed with MHC and cTnT staining following treatment. (mean ± SD, n=3) (C) Western blot for D3 (ES) cell samples collected on Day 6 and on Day 8. Cell extracts were probed for GATA-4 with β-tubulin as internal control. –(U) represents undifferentiated D3 (ES) cells and –(A) untreated and aggregated D3 (ES) cells. All quantification was normalized to undifferentiated control.

HAT Recruitment to the GATA-4 Promoter during cardiomyogenesis

EC cells were aggregated for 4 days in the presence of the inducer DMSO, with p300/ CBP HAT activity inhibited either during the early Day 0-2 or late Day 2-4 using Curcumin treatment. It was seen that p300 occupancy was decreased by over 7 fold following Curcumin treatment between Day 0-2 at the GATA-4 promoter (Figure 6A p300 ChIP panel). However, CBP did not have high occupancy at the GATA-4 promotor even following induction with DMSO, and the inhibition of AT activity at the early stage (Day 0-2) did not produce much change to its occupancy either (Figure 6A CBP ChIP panel).

Western blot analysis showed the presence of p300 and CBP (Figure 6B). Although the occupancy of p300 is largely reduced at the GATA-4 promoter during early HAT activity inhibition between Day 0-2, both p300 and CBP are is still expressed in the cell at the protein level (Figure 6A p300 ChIP panel, Figure 6B). β-tubulin was used as the loading control in the western blot studies.

Given the importance of histone acetylation in gene expression, we attempted to investigate the mechanism of AT activity at the GATA-4 locus by studying the effect of p300 recruitment on the histone acetylation at the GATA-4 locus

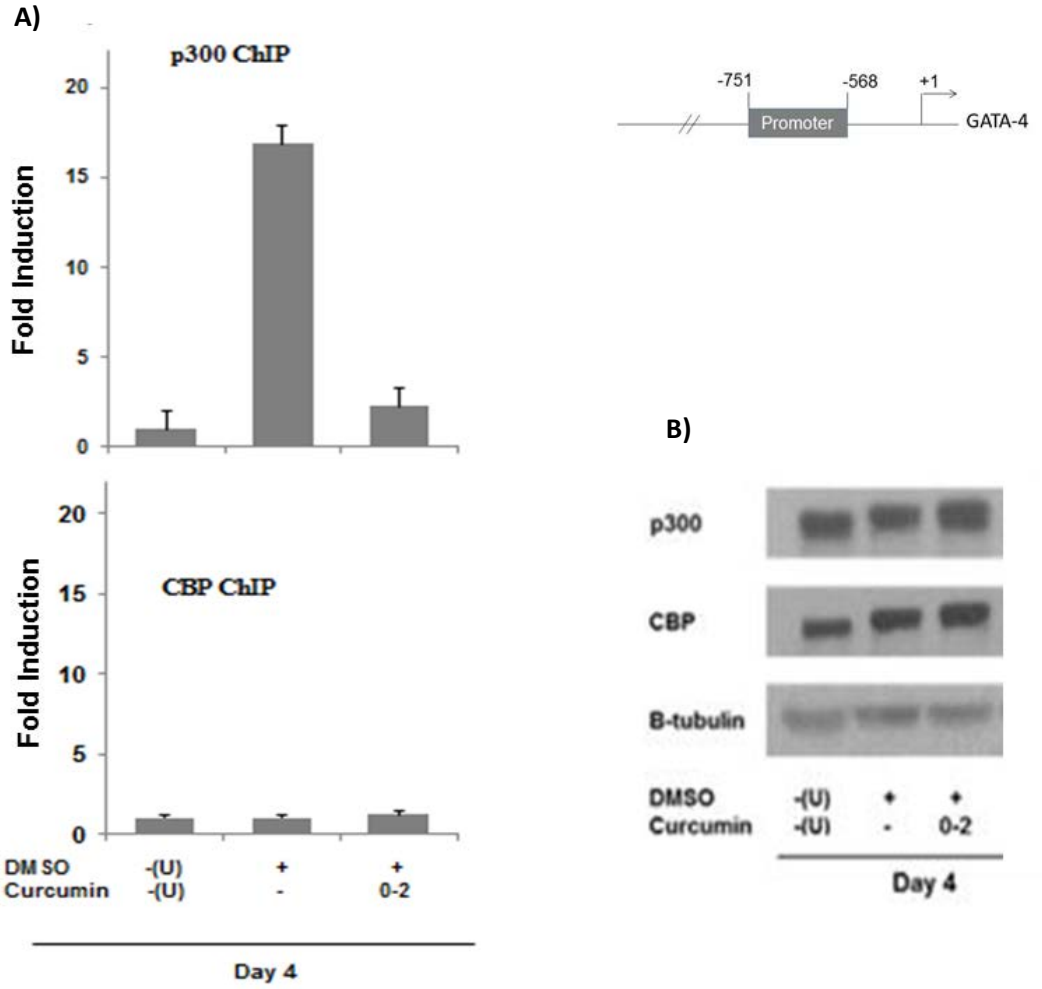

Figure 6. Early inhibition of HAT activity decreases p300 occupancy at the GATA-4 promoter. EC Cells were aggregated for 4 days treated with DMSO (0.8%), and inhibited with Curcumin (10µM) either between Day0-2 or Day 2-4. Cells were then cross-linked with 1% PFA. A) Relative change in p300 and CBP occupancy following treatment Values were normalized to undifferentiated control following RT PCR with GATA-4 promoter (-568bp to -751bp) primers. B) Western blot analysis for p300 and CBP expression with β-tubulin used as the internal control.

The effect of p300* H*AT Activity on Histone Acetylation at the GATA-4 Promoter

EC Cells were ChIPed with Histone 3 acetylated lysine. The acetylation of H3 had a 3 fold increase with DMSO treatment at the GATA-4 promoter in comparison to undifferentiated negative controls (Figure 7C), this correlated with the increase in p300 occupancy at the promoter (Figure 7D). When differentiating P19 EC cells were treated with Curcumin during the early first two days, there was an 8 fold decrease in p300 occupancy with a 2 fold decrease in the Histone 3 acetylation at the GATA4 promoter (Figure 7 C and D). Western blot analysis revealed a 2 fold decrease in the presence of acetylated histone 3 protein globally with Curcumin treatment during the early commitment stage of cardiomyogenesis, despite the expression of the p300 protein remaining fairly the same despite the inhibition of its AT activity with Curcumin (Figure 7 B and E).

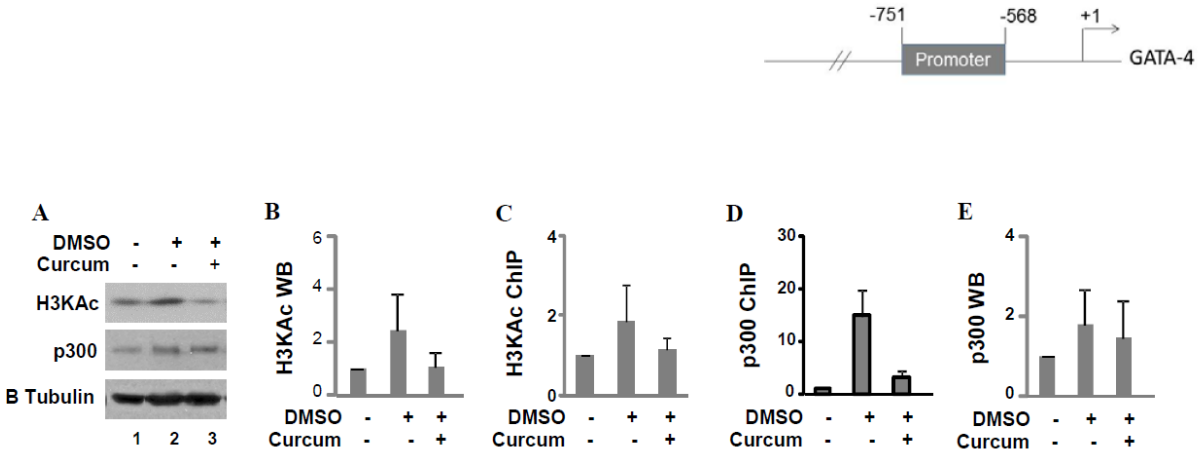

Figure 7. Recruitment of p300 and HAT activity during early commitment is important for Histone 3 acetylation at the GATA-4 promoter. EC Cells were aggregated for 4 days treated with DMSO (0.8%), Curcumin (10μM) as indicated above on Day 0-2 and cross-linked with 1% Paraformaldehyde (PFA). Western blots with H3KAc and p300 antibodies. β-tubulin was used as the loading control. Samples were ChIPed with H3KAc and p300 antibodies. (B) Western quantification of acetylated H3 represented as fold variations of the undifferentiated negative control (mean ± SD, n=3). (C) ChIP analysis of H3KAc at the GATA4 promoter represented as a fold variation of the undifferentiated control. (D) ChIP analysis of p300 occupancy at the GATA-4 promoter as determined by ChIP. (E) Western quantification of p300 protein as a fold variation of the undifferentiated control (mean ± SD, n-3) Real Time Polymerase Chain Reaction (RT PCR) analysis of the GATA-4 promoter was with primer (-568 to -751).

Negative ChIP Control

In this study, IgG antibody specific to the source animal of the experimental antibodies was also used as internal control for the ChIP experiments. The enrichment of IgG ChIP indicates a low background.

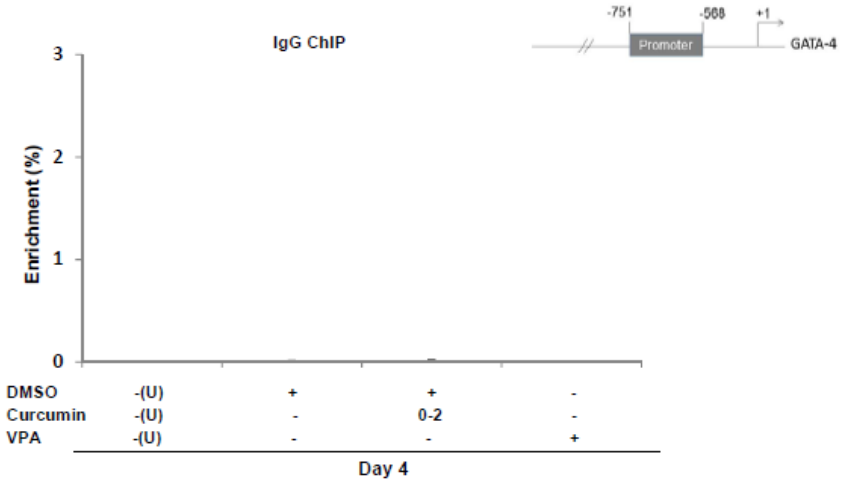

Figure 8. Negative IgG control for ChIP experiments. EC Cells were aggregated for 4 days treated with DMSO (0.8%), Curcumin (10μM) (Day 0-2), or VPA (0.5mM) as indicated above and cross-linked with 1% Paraformaldehyde (PFA). Samples were ChIPed with respective IgG antibodies. Values were normalized to undifferentiated control following Real Time Polymerase Chain Reaction (RT PCR) with GATA-4 promoter (-568 to -751).

DISCUSSION

DMSO is a more efficient inducer of cardiomyogenesis

In this study, EC and ES cells were used to study the role of HAT in cardoiomyogenesis. The differentiation of EC cells into cardiomyocytes was established using 0.8% DMSO. Previous studies have shown that treatment with 0.5-1% DMSO induced P19 cells to differentiate into skeletal and cardiac myocytes (Skerjanc, 1999); with 0.8% being commonly used to study cardiomyogenesis ((Wilton & Skerjanc, 1999); (Gianakopoulos & Skerjanc, 2009)). Following DMSO treatment, 10-25% of P19 EC cells differentiate into cardiomyocytes as observed with Myosin Heavy Chain staining (Skerjanc, 1999), our efficiency of 12.14% is consistent with these findings (Figure 3B).

It has also been known that HDAC inhibitors play a role in inducing differentiation of pluripotent cells into different lineages (reviewed in (Kretsovali, Hadjimichael, & Charmpilas, 2012)). HDAC inhibitors resemble the acetyl lysine substrate. For instance, the HDAC inhibitor TSA mediates its action via a metal binding group that coordinate a zinc ion to the active site of HDACs, a capping group that interacts with the residues near the entrance of the active site, and a linker region composed of hydrocarbon chains to coordinate the metal binding and capping groups. Class I and II HDACs are none specifically inhibited by TSA, while HDAC class III remains unaffected, as it does not function by an ion dependent mechanism (Bieliaukas & Pflum, 2008). TSA treatment of ES cells was found to decrease the expression of the pluripotency markers Nanog, Oct4, Sox2 as well as mediating a global increase in Histone 3 acetylation levels (Karantzali et al., 2008). Similarly, VPA and TSA were also found to increase global Histone 3 acetylation, but decrease the pluripotency marker FgF4 in the more stem F9 cells than the 19 cells (Shi, Gao, & Jin, 2011). In another study, TSA induced P19 EC differentiation by

increasing the expression of cardiac specific gene program consisting of Nkx2-5, MEF2C, and GATA-4 (Karamboulas et al., 2006).

Given these findings we decided to compare the efficiency of cardiomyogenesis with DMSO and VPA treatment, a more physiologic molecule that is widely used therapeutically as mentioned earlier. Our findings show that DMSO induces cardiomyogenesis with a higher efficiency than VPA as observed with both MHC and cTnT staining at a morphological level (Figure 3A and B). Given these, we would have expected higher levels of the cardiac differentiation marker expression following DMSO treatment. Although DMSO treatment resulted in higher occupancy at the GATA-4 promoter site than VPA treated EC cells (Figure 3D), and concomitant higher levels of GATA-4 expression with VPA treatment Figure 3D), this did not translate to higher efficiency in cardiomyogenesis with VPA treatment as seen in Figure 3A and B. This indicates that although gene expression is an indication of the commitment to the cardiac lineage, it does not necessarily translate into differentiation at a morphological level as elucidated by cardiac cell specific staining. This could potentially be due to the difference in the mechanism of lineage specification used by these two inducers, or the limitation of the choice of the proteins for cell staining to determine the efficiency. In any case, the percentage of cardiomyocytes observed with MHC staining was higher than cTnT staining. This many in part be due to MHC being expressed earlier in the cell, with cTnT expressed by the more mature cells later in differentiation. Since differentiation is a dynamic process there are cells present at different stages of differentiation within a given population at any time point of differentiation, although majority of the cells will be at the same developmental point; this in turn may account for the higher efficiency in MHC treatment than cTnT (Figures 3B, 4B, and 5B). In our study, we have chosen DMSO to continue forward with our investigation of HAT activity in cardiomyogenesis due to its higher efficiency of cardiomyocytes generated.

Next, we investigated the effect of histone acetyltranferase activity on cardiomyogenesis during different stages of cell commitment.

Early HAT inhibition impairs cardiomyogenesis in EC cells

Curcumin was used to inhibit the HAT activity of p300/CBP. Curcumin specifically inhibits HAT activity of p300/CBP, but the HAT activity of PCAF and methyltransferase and HDACs are also unaffected, both in vivo and in vitro (Balasubramanyam, Varier, Altaf, Swaminathan, Siddappa, Ranga, & Kundu, 2004a). In our study, we found that HAT activity in the early commitment stage is important during cardiomyogenesis in EC cells, such that the percentage of cells observed by immunofluorescence reduced to 1.25% (MHC) and 0.1% (cTnT) with inhibition of HAT activity from Day 0-2 (Figure 4A and B). However, the efficiency of cardiomyogenesis was affected to a lesser extent if the HAT activity was inhibited during the later stages of differentiation (Day 2-4) 9% (MHC) and about 7% (cTnT) (Figure 4A and B).

Given these findings, we performed western blots to investigate whether p300 HAT activity had a temporal role in the expression of the cardiac marker gene GATA-4. In keeping with the efficiency of differentiation, GATA-4 expression was highly reduced following HAT inhibition on Day 0-2 on Day 4 (Figure 4C and D). This was not a delay in GATA-4 expression, as on Day 7, the GATA-4 expression was not regained, unlike with late inhibition with Curcumin (Figure 4C and D). These findings indicate the HAT role of p300 or CBP plays an important role in the early cardiac lineage commitment mediated by GATA-4 expression.

Early HAT inhibition impairs cardiomyogenesis in ES cells

We also used ES cells to validate our findings in the EC cells, as ES cells are developmentally closer to actual embryonic development. Similar to our findings with P19 cells, inhibition of HAT activity with Curcumin treatment inhibited differentiation progressively to a lesser extent from Day 0-2 (3.73%), Day2-4 (7.87%), and Day 4-6 (11.93%) cTnT (Figure 5A and B). The results are similar with MHC staining (Figure 5B) but with higher overall efficiency as with P19 cells. Similarly, we investigated the expression of GATA-4 at the protein level using western blots in these cells (Figure 5C). These results are also in keeping with our EC findings (Figure 4C). However, the early inhibition decreases the expression of GATA-4, but does not necessarily suppress it as drastically as in P19 EC cells. It seems as though the ES cells are more resistant to the Curcumin insult. These results taken together with the findings from the P19 experiments indicate that AT activity is important in the lineage commitment of pluripotent cells and necessary for GATA-4 expression; however, additional compensatory mechanisms may play a role in the increased resilience of ES cells to HAT activity inhibition.

Recruitment of p300 to the GATA-4 promoter is affected by HAT activity

Inhibition of HAT activity during the early commitment stage to the cardiac lineage decreases p300 occupancy at the GATA-4 promoter (Figure 6A). This finding when taken into account with the IMF and western blot studies indicate that if p300 occupancy is reduced at the GATA-4 promoter during the critical commitment stage, GATA-4 expression is decreased (Figure 5C) as well as cardiomyogenesis (Figure 5A and B).

Since CBP HAT activity is also inhibited with Curcumin, we investigated whether there was any effect on CBP occupancy at the GATA-4 promoter. It was found that CBP did not have much occupancy at the GATA-4 promoter to begin with, and neither DMSO or Curcumin

produced significant change in CBP recruitment (Figure 6A), despite both proteins being present in the cell (Figure 6B). This difference in p300 and CBP activity is consistent with recent literature indicating that these two HATs have different roles in the cell, as discussed earlier. Moreover, these results are in keeping with other studies that found p300 to be more involved in cardiomyogenesis than CBP. For instance, it has been shown that p300 increases the DNA binding ability and stability of GATA-4, a key molecule in the cardiac lineage specification cascade ((Kawamura et al., 2005); (Dai & Markham, 2001)).

However, we can't say with certainty which of the two HATs has increased binding, and more experiments are needed to separate these out and the role of other HATs such as PCAF/GCN5.

Effect of HAT activity on histone acetylation at the GATA-4 promoter

The HAT p300 acetylates all Histone2A and Histone2B lysine residues. However, selectively acetylates lysine residue 18 of Histone 3 (Schiltz et al., 1999). Induction with DMSO resulted in increased p300 recruitment to the GATA-4 promoter and global increase in H3 acetylation (Figure 7A and D). Consistent with this, p300 recruitment to the GATA-4 promoter was associated with total Histone 3 acetylation at the GATA-4 promoter (Figure 7C and D); Although inhibition of HAT activity decreased p300 occupancy, and reduced the global H3 acetylation in the cell, the reduction in H3 acetylation was not as pronounced (Figure 7A, B, C, and D). This may in part be due to the acetylation by other factors such as P/CAF, as it is not inhibited by Curcumin (Balasubramanyam, Varier, Altaf, Swaminathan, Siddappa, Ranga, & Kundu, 2004b).

However, it would be interesting to see if these results can be replicated using H3 lysine 18 ChIP or if the acetylation of other H3 lysine residues are affected. Overall, it seems that p300 HAT activity is important for its recruitment to the GATA-4 promoter.

Mechanistically, it is known that the GATA-4 promoter has an Nkx2-5 binding site that is involved in GATA-4 expression during the early stages of cardomyogenesis (Riazi et al., 2009). It has also been shown that p300 interacts with Nkx2-5, SRF, and GATA-4 interacts with Nkx2-5 (He et al., 2011). Therefore, it is possible that inhibition of p300 HAT activity impairs its ability to interact with the Nkx2-5 cofactor and bind to the GATA-4 promoter, thereby inhibiting its expression. However, Curcumin may also have effects on other signaling pathways that indirectly also affect cardiomyogenesis including the suppression of the transcription of Stat3 (Bharti, Donato, & Aggarwal, 2003), inhibition of the Wnt/β catenin pathway by increasing the degradation of β catenin by Caspase 3 distinct from that resulting by GSK3β phosphorylation ((Jaiswal, Marlow, Gupta, & Narayan, 2002); (Park et al., 2005)).

In the future, it would be interesting to investigate using IMF techniques as well as western blotting to study whether the inhibition of HAT activity during the early stages of cardiomyogenesis triggers the signaling cascades of other lineages. Moreover, Fluorescence-Activated Cell Sorting (FACS) studies can be used to elucidate the type of cardiac cells produced by DMSO and VPA induction. It would be interesting to see if different types of cells are generated by the two inducers, or whether the inhibition of one pathway opens a gateway into another. Moreover, the influence of HAT inhibition on different cardiac cell types, if any, remains yet to be studied. In addition to this, it would be important to perform ChIP experiments to investigate whether P/CAF and GCN5 have differential binding roles at the GATA-4 locus. Additionally, the transcription factor(s) responsible for the recruitment of p300 to the GATA-4

locus is still unknown, as well as the effect of HAT activity on this said transcription factor(s) if any.

CONCLUSION

Histone acetyltransferase activity is important for the expression of GATA-4 and cardiomyogenesis during the early commitment stage to the cardiac lineage. This is possibly mediated by temporally specific, histone acetyltransferase dependent, recruitment of p300 to the GATA-4 promoter and histone acetylation during the early commitment stage.

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