MECHANISMS OF VALPROIC ACID-INDUCED LIMB MALFORMATIONS IN THE MURINE LIMB BUD CULTURE SYSTEM

By

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Abstract

In utero exposure to valproic acid (VPA), an anticonvulsant and antidepressant, induces neural tube and skeletal malformations through a molecular mechanism that remains unsolved. VPA inhibits histone deacetylases (HDACs) whereas its very close structural analog, valpromide (VPD), does not. Although HDACs are separated into four classes, all HDACs share common targets and it is not known if they play distinctive roles during limb development. Limb pattern formation involves several well characterized signaling pathways that drive cellular processes, including chondrocyte differentiation, bone mineralization and interdigital apoptosis. My goal is to test the hypothesis that VPA causes limb malformations by inhibiting HDACs, leading to altered regulation of cell differentiation and death during limb development.

My first objective was to characterize the effects of VPA and VPD on chondrogenesis and osteogenesis in a limb bud culture system by determining their effects on the expression of specific markers of these processes. Both Sox9/chondrogenesis and Runx2/osteogenesis signaling were downregulated in VPA but not in VPD-treated limbs, suggesting an HDAC dependent mechanism.

The second objective was to examine the effects of VPA on p53 signaling and cellular apoptosis in limbs and to investigate the impact of HDAC inhibition on this process. VPA treatment induced the hyperacetylation and activation of p53, triggering the intrinsic apoptotic pathway leading to the activation of caspase 3. VPD had no significant effect on this pathway.

My final objective was to investigate the role of different HDAC classes in

chondrogenesis and osteogenesis; I used class-specific pharmacological inhibitors and triple transgenic mice expressing markers of chondrocyte and osteoblast differentiation combined to a fluorescent tag. Exposure to VPA or a class I inhibitor had a rapid effect on both chondrogenesis and osteogenesis markers. In contrast, exposure to a class II inhibitor had a slight effect on osteogenesis differentiation only, whereas the class III inhibitor caused a very late drastic effects on the whole limb. Changes in morphology in limbs exposed to class I and III inhibitors were also correlated with an increase in apoptosis, suggesting that both cellular differentiation and death are pivotal for limb development.

Collectively, these results show that VPA acts on multiple processes crucial for limb development and demonstrate a correlation between HDAC inhibition and teratogenesis.

Résumé

L'exposition intrautérine à l'acide valproique (VPA), un anticonvulsivant et antidépresseur, cause des malformations du tube neural et du skelette par un mécanisme moléculaire inconnu. VPA est un inhibiteur des histones désacétylases (HDAC) alors que son analogue structural, valpromide (VPD), ne l'est pas. Bien que les HDACs soient séparées en quatre classes, ils partagent les même cibles moléculaires et leur rôle dans le développement des membres antérieurs est inconnu. La formation des membres implique plusieurs voies de signalisation bien caractérisés, qui controlent divers processus cellulaires y compris la différenciation des chondrocytes, la minéralisation osseuse et l'apoptose interdigitale. Mon but est de vérifier l'hypothèse que le VPA provoque des malformations des membres en inhibant les HDACs, menant à une modification de la régulation de la différenciation et de la mort cellulaire pendant le développement.

Mon premier objectif était de caractériser les effets du VPA et du VPD sur la chondrogenèse et ostéogenèse dans un système de culture de membre *in vitro* et de déterminer leurs effets sur l'expression des marqueurs spécifiques de ces processus. Les voies de signalisation Sox9/chondrogenèse et Runx2/ostéogenèse ont été réprimés par l'exposition au VPA, mais pas au VPD, suggérant un mécanisme dépendant des HDACs.

Le second objectif était d'examiner les effets du VPA sur les voies de signalement p53 et l'apoptose dans les membres et d'étudier l'impact de l'inhibition des HDAC sur ce processus. Le VPA induit l'hyperacétylation et l'activation de p53, déclenchant la voie

apoptotique intrinsèque menant à l'activation de la caspase 3. VPD n'eut aucun effet significatif sur cette voie.

Mon objectif final était d'examiner le rôle des différentes classes d'HDAC sur la chondrogenèse et ostéogenèse; j'ai utilisé des inhibiteurs pharmacologiques spécifiques au classe et des souris transgéniques exprimant des marqueurs de différenciation des chondrocytes et des ostéoblastes, combinée à une protéine fluorescente. L'exposition au VPA ou l'inhibiteur de la classe I des HDACs eut un effet rapide sur les marqueurs de chondrogenèse et de l'ostéogenèse. En revanche, l'exposition à un inhibiteur de HDAC classe II a un léger effet sur la différenciation de l'ostéogenèse seulement, alors que l'inhibiteur de la HDAC classe III cause des effets drastiques sur l'ensemble du membre. Les changements dans la morphologie dans membres exposés aux inhibiteurs de la classe I et III étaient également corrélées avec une augmentation de l'apoptose, ce qui suggère que les deux processus de différentiation et la mort cellulaire jouent un rôle essentiel pour le développement des membres.

Collectivement, ces résultats démontrent que le VPA agit sur plusieurs processus cruciaux pour le développement des membres et démontrent une corrélation entre l'inhibition des HDACs et la tératogenèse.

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List of Abbreviations

8-OHdG	8-hydroxyguanosine
Act D	Actinomycin D
AD	Activation domain
AER	Apical ectodermal ridge
ALDH5A1	Succinate semialdehyde dehydrogenase
ALP	Alkaline phosphatase
AML	Acute myeloid leukaemia
APAF	Apoptotic-protease-activating-factor 1
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BCL2	B-Cell Lymphoma 2
BMP	bone morphogenetic protein
CBF	Core-binding factor
CBP	CREB-binding protein
CCD	Cleidocranial dysplasia
CDK1	Cyclin-dependent kinase 1
CHK2	Checkpoint kinase 2
COL10A1	Collagen type 10 a1
COL1A1	Collagen type I a1
COL2A1	Collagen type 2 a1
CpG	Cytosine-guanine

CREB	cAMP response element-binding protein
CYP	Cytochrome P450
DMNT	DNA methyltransferases
eCFP	Enhanced cyan fluorescent protein
EZH2	Enhancer of zeste homolog 2
FADD	Fas-associated protein with death domain
FGF	Fibroblast growth factor
FGFR	FGF receptor
GABA	Gamma amino butyrate
GABAT	GABA transaminase
GCN5	general control of amino-acid synthesis 5
GD	Gestational day
GLI3	GLI family zinc finger 3
GLI3R	GLI3 repressor
HAT	Histone acetyltransferases
HATi	HAT inhibitor
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HIF	Hypoxia-inducible factor
HMG	High-mobility-group
HRP	horseradish peroxidase
IAP	Inhibitors of apoptosis protein
IC50	Half maximal inhibitory concentration

IKKα	Inhibitor of kappa kinase α
LMX1	LIM homeobox transcription factor 1
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MMP	Matrix metalloproteinase
MTHFR	Methylenetetrahydrofolate reductase
N-CAM	Neural cell adhesion molecule
NES	Nuclear export signal
NF-kB	RelA/Nuclear factor kB
NKX3.2	NK3 homeodomain factors 2
NLS	Nuclear localization signal
NMTS	Nuclear matrix targeting signal
NTD	Neural tube closure defect
p21	Cyclin-dependent kinase inhibitor 1
PARP	Poly (ADP-ribose) polymerase
PBN	N-tertiary-butyl nitrone
PCAF	P300/CBP-associated factor
PEBP	Polyoma enhancer-binding protein
PPAR	Peroxisome proliferator-activated receptors
PRC	polycomb repressor complex
PRX1	Paired related homeobox 1
PST	Proline serine threonine domain
PTHR	Parathyroid hormone receptor

PTHrP	Parathyroid hormone-related protein
PVDF	Polyvinylidene fluoride
PZ	Progress zone
QA	Glutamine-alanine domain
qRT-PCR	Quantitative Reverse transcription polymerase chain reaction
RHD	Runt homology domain
RUNX2	Runt-related transcription factor 2
SHH	Sonic-hedgehog protein
SIRT	Sirtuin
SIRTi	Sirtuin inhibitor
SOX9	SRY-box 9
SRY	Sex determining region Y
TAD	Transactivation domain
TBX	T-box
TGFβ	Transforming growth factor beta
TIP60	Tat interactive protein 60
UGT	UDP glucuronosyltransferase
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
VPD	Valpromide
WNT	Wingless
YFP	Yellow fluorescent protein
ZPA	Zone of polarizing activity

γH2A.X	Phosphorylated histone variant H2A.X

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Preface

Format of the Thesis

This is a manuscript-based thesis in accordance with the Thesis Preparation and Submission Guidelines outlined by the Faculty of Graduate and Postdoctoral Studies at McGill University.

This thesis consists of five chapters. Chapter 1, the introduction, provides a review of main concepts explored in the studies presented in this thesis; the pharmacology and teratology of valproic acid, epigenetics and the processes and affiliated pathways involved in limb formation. The hypothesis and specific research objectives are also presented in Chapter 1. Chapter 2, 3, and 4, are data chapters and are separated by connecting texts highlighting the links between the consecutive chapters. All together, these chapters addressed my hypothesis. Chapter 2 is published in Toxicological Sciences (2013 Jan;131(1):234-41). Chapter 3 and 4 are manuscripts in preparation. A summary, overall discussion, final conclusions, and future directions are presented in Chapter 5. A list of original contributions is also found at the end of this chapter. References are provided at the end of the thesis.

Contribution of Authors

All the experiments presented in this thesis were performed by the candidate.

Chapter 1 General introduction

1.1 Valproic acid (VPA)

Valproic acid (2-propyl-pentanoic acid) is a short-chain fatty acid derived from valeric acid, found in the roots of the valerian plants. It was first synthesized in 1882 and was initially used as an organic solvent which led to the serendipitous discovery of its anticonvulsant effects in 1963 (Burton 1882, Meunier, Carraz et al. 1963). The first clinical trials using valproic acid were reported in 1964 (Carraz, Fau et al. 1964). It was marketed in France in 1967 and released in the United States in 1978. By the late 1970s, valproic acid was marketed worldwide and attained the status of a major antiepileptic drug. It is now marketed under the names Depakote, Depakene, Stavzor, Divalproex and Epival. Currently, VPA is used in the treatment of epilepsy, migraine prophylaxis and bipolar, mood, anxiety and psychiatric disorders (Evers 2008, Bowden 2009). It is now in preclinical trials as a chemotherapeutic agent and shows positive effects and therapeutic potential in animal models of Alzheimer's disease (Qing, He et al. 2008, Nebbioso, Carafa et al. 2012).

1.1.1 Pharmacokinetics and metabolism

Although it is available in rectal and injectable dosage forms, valproic acid is mostly given orally. It is quickly absorbed and the serum levels reach their maximum after 2 to 5 hours following the oral dose (Perucca 2002). VPA is 87-95% protein bound in the blood stream and hence has a very low clearance, with a mean half-life ranging from 9 to 16h (Bryson, Verma et al. 1983, Leppik and Birnbaum 2010). The therapeutic plasma concentrations range between 85 to 125ug/mL (0.50-0.75mM) for bipolar disorders and 50 to 100ug/mL (0.30-0.60mM) in cases of epilepsy (2014). During pregnancy, total

VPA concentrations decrease by 25 to 50%; however, the unbound VPA concentrations in plasma remain the same and concentrations up to 5 times higher than the therapeutic range were found in the fetal cord blood, suggesting that VPA bioaccumulates in the embryo during pregnancy (Vajda and Eadie 2005, Sabers and Tomson 2009). VPA has three main routes of metabolism in the liver (Fig.1.1A); it is mostly modified by glucuronidation by members of the UDP glucuronosyltransferases (UGT) family, leading to the formation of VPA-glucuronide, excreted in the urine (Argikar and Remmel 2009). β-Oxidation in the mitochondria is the second major contributor to VPA metabolism; the remaining 10% is metabolized by cytochrome P450 (CYP) enzymes, notably CYP2A6, CYP2B6, CYP2C19 and CYP2C9 (Ito, Ikeda et al. 1990, Fleming and Chetty 2005, Tan, Yu et al. 2010). VPA as well as some of its active metabolites, such as 2-n-propyl-4-pentenoic acid (4-ene-VPA), can induce toxicity; the most severe rare life-threatening side effects of VPA include hepatotoxicity and pancreatitis as well as teratogenicity (Siemes, Nau et al. 1993, 2014). The metabolism of VPA depends on a number of factors, including UGT and CYP variants and drug-drug interactions which cause very variable dosage requirements in adults (Yu, Shen et al. 1990, Blanco-Serrano, Otero et al. 1999, Krishnaswamy, Hao et al. 2005, Tan, Yu et al. 2010).

1.1.2 Pharmacodynamics and mechanisms of action

VPA has a wide range of targets that explain its different pharmacological uses (Fig.1.1B). VPA indirectly regulates gamma amino butyrate (GABA) levels in the brain by targeting

Figure 1.1: Schematic overview of valproic acid pharmacokinetic and pharmacodynamic pathways.

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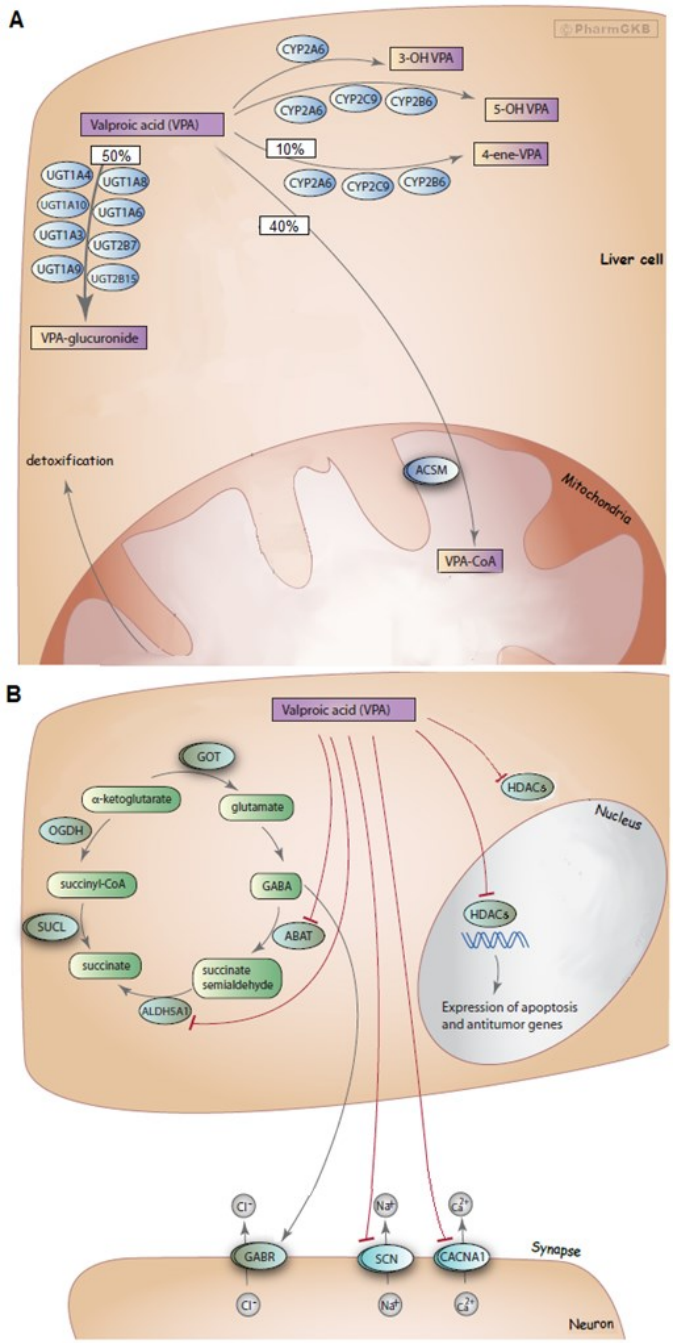


the GABA degrading enzymes GABA transaminase (GABAT) and succinate semialdehyde dehydrogenase (ALDH5A1) (Johannessen and Johannessen 2003). GABA is the main inhibitory neurotransmitter in the central nervous system and its increase is thought to be one of the main mechanism by which VPA prevents seizures. It also blocks voltage-gated sodium channels, thereby reducing excitatory neurotransmission (Zona and Avoli 1990, Van den Berg, Kok et al. 1993). VPA inhibits a family of enzymes called histone deacetylases (HDAC). More specifically, it inhibits class I and II HDACs (Gottlicher, Minucci et al. 2001, Phiel, Zhang et al. 2001).

1.1.3 Teratogenicity of valproic acid

VPA crosses the placenta and adversely affects the development of the fetus during pregnancy. It causes an array of minor or life-threatening birth defects that have been named the fetal valproate syndrome. Although migraines can be treated with alternative medications, to this date, no known effective anticonvulsant has been shown to be completely safe for the developing embryo (reviewed in (Wlodarczyk, Palacios et al. 2012)). On the other hand, a sub-efficient or a complete withdrawal from the drug can lead to status epilepticus episodes which could be detrimental to both the mother and the embryos (Goodwin and Lawson 1947). Therefore, management of epilepsy during pregnancy poses a very difficult dilemma. It is recommended to avoid polytherapy and reduce the dose to the lowest efficacious dosage. Recently, carbamazepine and lamotrigine have been increasingly used in woman of reproductive age; although these compounds are less potent that VPA as anticonvulsants, they have also been shown to be less teratogenic and mostly increase the risk of cleft lips and palate, two non-lethal

birth defects (Perucca 2005). Folic acid supplementation may help diminish the risk of antiepileptic drug-induced major congenital malformation however current studies on the subject are inconsistent. (Wlodarczyk, Palacios et al. 2012)

1.1.3.1 Animal and epidemiological studies

VPA has been extensively studied and its teratogenesis characterized in many animal model systems: zebrafish, *Xenopus*, chicken, mouse, rat, rabbit, rhesus monkey (Hendrickx, Nau et al. 1988, Gurvich, Berman et al. 2005, Riebeling, Pirow et al. 2011, Hsieh, Wang et al. 2012). These studies were done at different gestational times and with various doses and therefore showed a variety of defects, including craniofacial, cardiac, neural tube defects and, most commonly, skeletal defects. In non-mammalian vertebrates, VPA causes growth retardation, pigmentation defects, cardiac anomalies and axial malformations (Gurvich, Berman et al. 2005). Gross skeletal malformations are also observed in chicks and rhesus monkey and were especially prominent in rabbits (Petrere, Anderson et al. 1986, Hendrickx, Nau et al. 1988, Hsieh, Wang et al. 2012). In humans, VPA causes an array of specific malformations that are commonly referred to as the fetal valproate syndrome, first described by Di Liberti et al., and further divided into major congenital malformations and neurobehavioral cognitive defects (DiLiberti, Farndon et al. 1984). The former includes craniofacial features such as orofacial clefts, midface hypoplasia, epicanthal folds, a narrow bifrontal skull and a broad flat nasal bridge. Spina bifida, a type of neural tube closure defect (NTD) is the one of the most commonly observed congenital malformations in VPA-exposed children (Werler, Ahrens et al. 2011). VPA causes an increased risk of skeletal, cardiac and

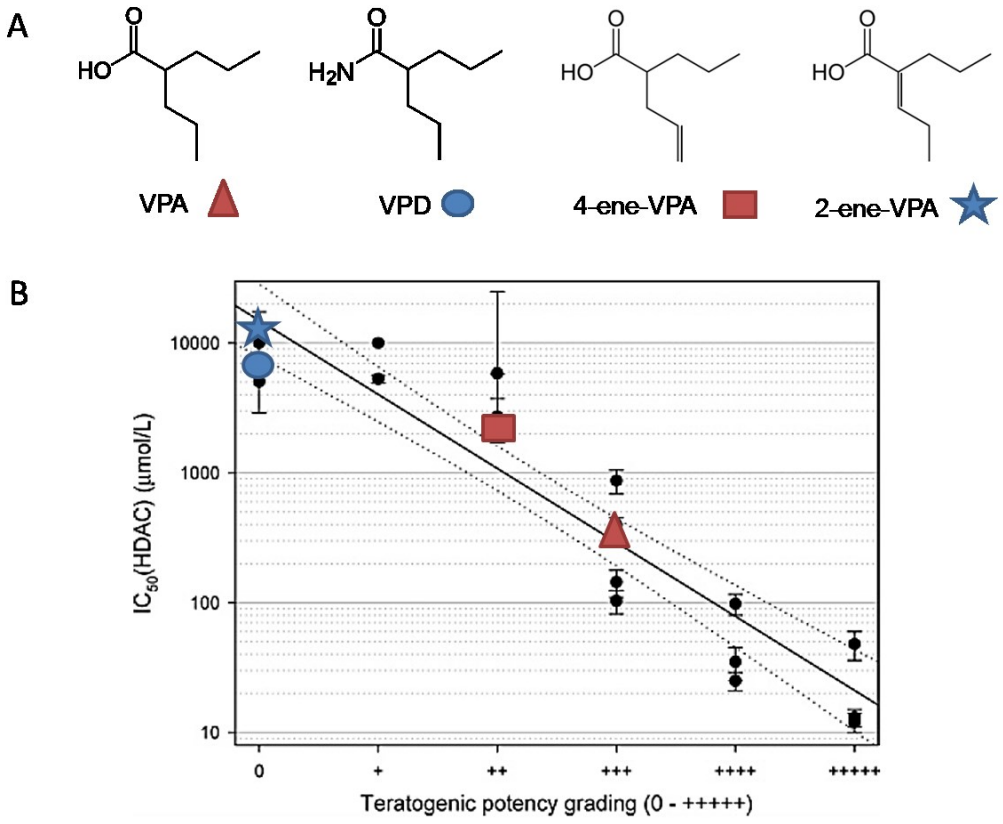
urogenital defects. VPA worldwide use during pregnancy has led to its inclusion in a number of a pregnancy registries and prospective studies. A study reported the pregnancy outcome of 715 children in the United Kingdom exposed to VPA monotherapy in utero; defects included: orofacial clefts (1.5%), NTDs (1%), skeletal defects (1.1%), cardiac malformations (0.7%), and urogenital defects (0.9%) (Morrow, Russell et al. 2006). A study in the North American pregnancy registry reported major congenital malformations in 10.7% of those women receiving VPA monotherapy, as opposed to 2.9% of those who received other anticonvulsants (Wyszynski, Nambisan et al. 2005). More recently, in utero exposure to VPA has been shown to impair cognitive function, including developmental delays, lower verbal and non-verbal intelligence coefficient (IQ), impaired social behavior (Adab, Kini et al. 2004, Vinten, Bromley et al. 2009). Moreover, a high incidence of autism spectrum disorders is found in VPA-exposed children (Rasalam, Hailey et al. 2005). In response to these studies, the U.S. Federal Food and Drug Administration released safety alerts in 2009 and 2011 concerning the increased risks of neural tube defects, congenital malformations and impaired cognitive development associated with VPA exposure during pregnancy (FDA 2009, FDA 2011).

1.1.3.2 Structural analogs of VPA and mechanisms of teratogenicity

Various analogs of VPA were examined for their teratogenicity and used as comparative tools to establish the molecular mechanisms underlying its developmental toxicity. More specifically, the non-teratogenic 2-ene-VPA and the teratogenic 4-ene-VPA metabolites,

Figure 1.2 Valproic acid and its analogs

A. Molecular structure of valproic acid (VPA) and its analogs, valpromide (VPD), 2-propyl-4-pentenoic acid (4-ene-VPA) and 2-propyl-2-pentenoic acid (2-ene-VPA). B. Teratogenic potency of compounds as a function of their HDAC inhibition activity. Modified from (Eikel, Lampen et al. 2006)



were used in a number of studies (Fig.1.2A). It was established that a double bond on C2 or C3, a large alkyl group on C2 and the absence of a free carboxyl group led to a decrease or complete lack of teratogenicity. Only some of these changes affected the anticonvulsant capability, suggesting these two mechanisms are unrelated (Wlodarczyk, Palacios et al. 2012).

Altered folate metabolism was suggested as a possible mechanism of teratogenicity (Wegner and Nau 1992); folate has been shown to play a crucial role in the formation of DNA nucleotides and DNA methylation pathways and is critical for proper development (Nazki, Sameer et al. 2014). A study by Wegner and Nau showed that VPA causes a concentration dependant disruption of folate metabolism in mouse embryonic tissue and that these effects were not observed with the 2-ene-VPA analog (Wegner and Nau 1992). VPA upregulates the expression of methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in folate regulation. VPA induced teratogenicity was reduced in the folate-supplemented group and in MTHFR heterozygote (+/-) mice (Dawson, Raymond et al. 2006, Roy, Leclerc et al. 2008).

VPA increases reactive oxygen species thereby causing oxidative stress that was reported to play an important role in VPA-induced neural tube defects (Tung and Winn 2011). In addition, VPA activates peroxisome proliferator-activated receptors (PPAR) gamma and disrupts Wingless (WNT)/β-Catenin signaling, two important pathways involved in numerous developmental and cellular processes (Lampen, Carlberg et al. 2001, Wiltse 2005). However, the exact mechanism by which VPA affects these pathways remains unresolved. Finally, as mentioned previously, VPA directly inhibits

histone deacetylases (HDACs) and it has been hypothesized that this interaction is linked to its teratogenicity. Eikel et al. compared the teratogenic and HDAC inhibition potential of 20 different structural analogs of VPA and established a direct correlation between these two components (Fig.1.2B) (Eikel, Lampen et al. 2006). More specifically, both valpromide (VPD) and 2-ene-VPA have low teratogenicity and very high HDAC half maximal inhibitory concentration (IC50s), whereas the reverse is observed for VPA and 4-ene-VPA (Eikel, Lampen et al. 2006). Moreover, several other HDAC inhibitors, including trichostatin A, apicidin, sodium butyrate and boric acid, have been shown to cause axial skeletal defects as well as other malformations (Di Renzo, Broccia et al. 2007, Di Renzo, Cappelletti et al. 2007).

1.2 In vitro limb bud culture as a model to study developmental toxicity and teratogenicity

Over the past 30 years, there has been rising interest in finding alternatives to whole-animal experiments to do toxicological testing for a number of reasons; both the scientific community and the general public have increasing ethical and moral concerns with the use of high numbers of animals in large scale experiments. Moreover, as thousands of new chemicals are created and produced every year, the time and monetary resources required to do whole animal toxicological studies became a limitation as these are both lengthy and expensive. Finally, it is recognized that these whole-animal experiments often focus on describing toxicity, providing little information about the underlying mechanisms leading to these toxic effects (Neubert and Barrach 1977). Several models have been developed and used in developmental toxicology;

these range from invertebrate and non-mammalian vertebrate (worm, zebrafish, amphibians) to in vitro culture, (whole-embryo culture, organ culture, mouse embryonic stem cells (mESCs)) (reviewed in (2012)). Limb development has been used for many decades as a model to study organogenesis. Over the years, studies of limb bud development have provided invaluable insights into the molecular mechanism and common principles implicated in organogenesis, including ectoderm-mesoderm interactions. Some of the best examples of this process may be that of the grafting experiments done in chicks by Wessels in 1977 (Wessells 1977). These clearly demonstrated the roles of the apical ectodermal ridge (AER) and the underlying forelimb mesenchyme in the specification and identity of the limb as limb development ceases when either the AER was removed or the mesenchyme was replaced by nonlimb mesenchyme. Another example is the "ectodermal Wnt7a-mesenchymal Lmx1" signaling, leading to the establishment of the dorsal-ventral axis of the limb (described later in section 1.4.1). Moreover, the induction and specification principles are very well illustrated by fibroblast growth factor (FGF) 10-mediated induction of limb buds and the rostral-caudal T-box (TBX) 5-TBX4 gradient involved in the specification of forelimb/hindlimb identity; a FGF soaked-bead placed at different positions in the lateral plate mesoderm will yield a wing, a leg or a chimera or both, depending on the TBX factor presents (Ohuchi, Nakagawa et al. 1999).

The in vitro limb bud culture system has been used for decades as a tool to study developmental toxicology (Neubert and Barrach 1977). As is the case with all research models, this culture system has both its advantages and disadvantages; the culture in vitro allows for a very tight control over the culture conditions and exposures and

bypasses maternally-mediated effects. Also, limb bud cultures can be maintained for 6 days, whereas other in vitro cultures, such as primary cells or whole embryo cultures, usually start degrading after 24h. However, although some processes, such as mesenchymal condensation, cartilage and bone formation and interdigital apoptosis occur in these cultures, others processes are disrupted; limb innervation arises from migration of neural precursors into the limb at a later time and cannot be achieved after the limb is excised. Moreover, the blood supply is also disrupted although it is unclear whether limbs cultured in vitro form blood capillaries. Also, limbs cultured in vitro develop at a slower pace than those in vivo.

1.3 Epigenetics and histone modifications

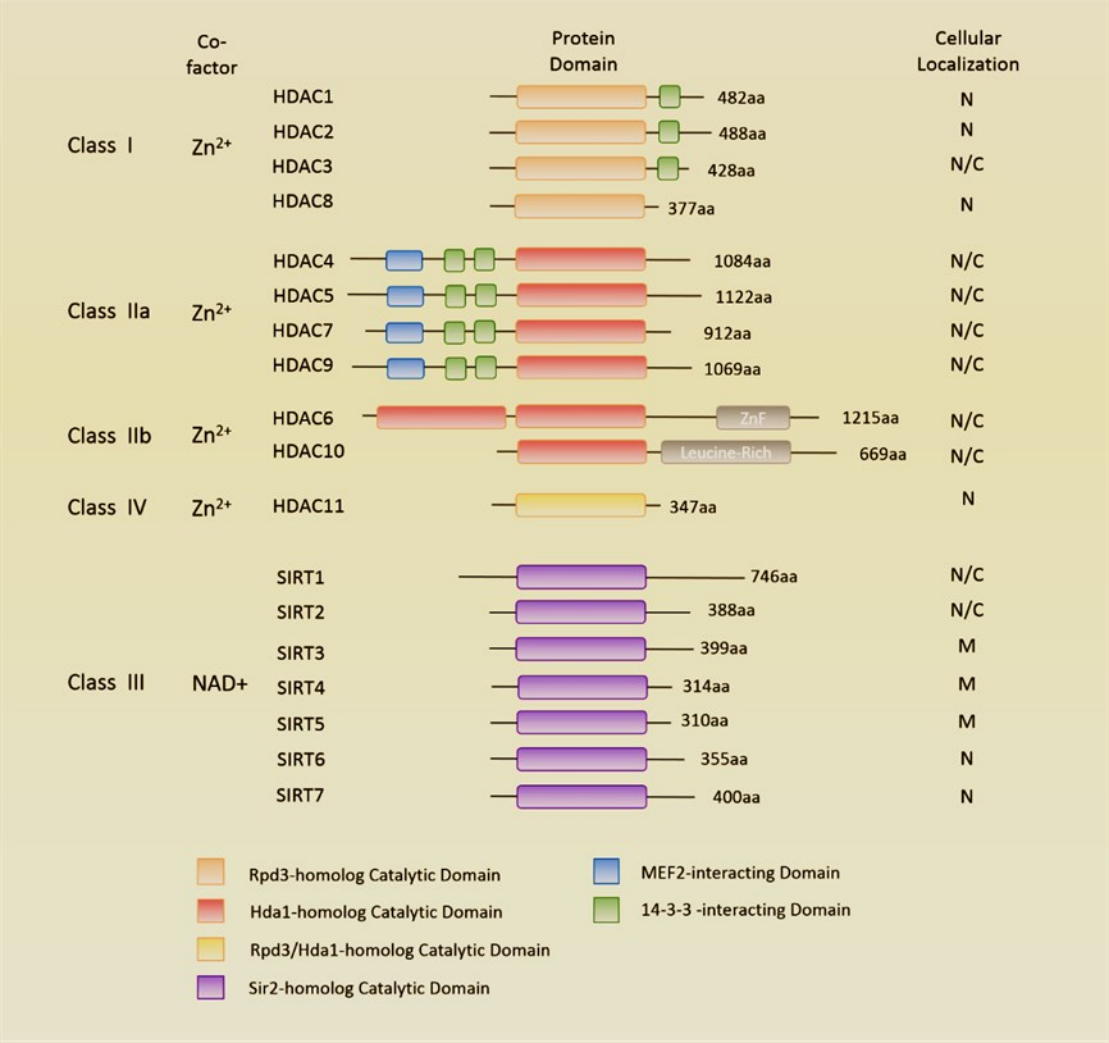
Regulation of cellular gene expression depends on both genetic and epigenetic mechanisms; whereas the former relies solely on the DNA sequence, the latter is a very complex network of reversible protein and DNA modifications that modulate the accessibility to the genome and therefore its expression. Epigenetic mechanisms include DNA methylation and histone modifications, notably histone acetylation and methylation. Several groups also consider transcription factors to be included in these mechanisms as they play a key role in regulating gene expression (Stein, Stein et al. 2010). The addition of a methyl group to the 5^{th} carbon of a cytosine residue within a cytosine-guanine (CpG) dinucleotide is achieved by DNA methyltransferases (DNMTs) (Turek-Plewa and Jagodzinski 2005, Doerfler 2008). It is associated with steric hindrance of the promoter and a repression of gene expression (Derreumaux, Chaoui et al. 2001, Stengel, Fiebig et al. 2010). Histone tails are also commonly methylated by

lysine methylating enzymes and complexes such as polycomb repressor complex (PRC1/2) (Kim, Paylor et al. 2006). Histone acetylation is a reversible posttranslational modification, which alters chromatin compaction and leads to changes in gene expression; acetylation of lysine residues modifies the ionic charge of the histone tails toward a more neutral charge, which leads to a weaker interaction with the negatively charged phosphate group of the DNA and a more open chromatin structure (Bannister and Kouzarides 2011). Histone acetyltransferases (HATs) such as p300/CREB-binding protein (CBP), P300/CBP-associated factor (PCAF), TAT interactive protein 60 (TIP60) and general control of amino-acid synthesis 5 (GCN5), are the enzymes responsible for the addition of acetyl groups on the histone tails whereas histone deacetylases (HDACs) are involved in their removal (Davie and Spencer 1999).

1.3.1 Histones deacetylases (HDACs)

HDACs are divided into different classes based on similarity to their *S. cerevisiae* homologs (Fig.1.3); classes I, II and IV are commonly referred to as HDACs or traditional HDACs whereas class III HDACs are known as Sirtuins. Class I HDACs (HDAC1, -2, -3, and -8) are nuclear proteins homologous to the yeast RPD3 and are mostly ubiquitously expressed in different tissues. Class II HDACs are homologous to the yeast HDA1 histone deacetylase and are further divided into class IIa (HDAC4,-5, -7, and -9) and IIb (HDAC6 and -10) depending on their respective domains: Class IIa HDACs have a very conserved C-terminal catalytic domain and a more variable N-terminal domain, whereas class IIb HDACs are characterized by the presence of two

Figure 1.3 HDAC classes and their cofactor, protein domains and cellular localisations



tandem HDAC domains. HDACs from both of these classes shuttle between the nucleus and cytoplasm and exhibit tissue specific expression in adults (Reviewed in (Verdin, Dequiedt et al. 2003, Clocchiatti, Florean et al. 2011). HDAC4, -5, and -9 show high expression in the heart, brain and skeletal muscle, HDAC7 is highly expressed in heart and lungs, whereas HDAC10 is expressed mostly in spleen, liver and kidney and HDAC6 is found in several neural tissues (Magdaleno, Jensen et al. 2006). More recently, class IV, which is comprised of HDAC11, has been recognized; its catalytic domain shares similarities with both RPD3 and HDA1 and little is known of its expression pattern. Finally, Class III HDACs consist of the sirtuins (SIRT1-7), which are NAD+-dependent enzymes and are homologous to the yeast SIR2. SIRT1, -6 and - 7 are mostly nuclear. SIRT2 shuttles between the nucleus and cytoplasm and SIRT3, 4 and 5 are mitochondrial proteins (Villalba and Alcain 2012).

1.3.2 Epigenetic regulation during development

Epigenetics have been shown to regulate the pluripotency and differentiation of stem cells, the maintenance of imprinted genes, and X-chromosome inactivation and are therefore pivotal for early embryonic development (Fedoriw, Mugford et al. 2012, Boland, Nazor et al. 2014, Kamikawa and Donohoe 2014). In addition, several knockout studies have provided valuable insights into the role of epigenetic marks during organogenesis that will be further discussed in the next section (Table 1).

1.3.2.1 HATs and histone methyltransferase complex

Many of the double allele knockout mice of histone modifying enzymes exhibit embryonic lethality at the organogenesis stage, suggesting a major role for these

Table 1.1 Summary of transgenic knockout mouse studies of HDACs, SIRTs and other epigenetic enzymes

Genotype	Phenotype	Ref
Hdac1 -/-	Embryonic lethal GD9.5-10.5, severe growth retardation and proliferation defects	(Montgomery, Davis et al. 2007)
Hdac2 -/-	Die perinatally, ventricular cardiac defects	(Montgomery, Davis et al. 2007)
Hdac3 -/-	Embryonic lethal before GD9.5	(Bhaskara, Chyla et al. 2008)
Hdac4 -/-	Viable, runted, premature chondrocyte hypertrophy	(Vega, Matsuda et al. 2004)
Hdac5 -/-	Viable, enlarged heart	(Chang, McKinsey et al. 2004)
Hdac6 -/-	Viable, increased tubulin acetylation	(Zhang, Kwon et al. 2008)
Hdac7 -/-	Embryonic lethal GD11, major vascular system defects	(Chang, Young et al. 2006)
Hdac8 -/-	Die perinatally from brain hemorrhage , skull bone ossification defects, major growth retardation	(Haberland, Mokalled et al. 2009)
Hdac9 -/-	Viable, enlarged heart	(Chang, McKinsey et al. 2004)
Sirt1 -/-	Embryonic lethal GD9.5-14.5, growth retardation, chromosome abnormalities	(Wang, Sengupta et al. 2008)
Sirt2 -/-	Viable, chromosome abnormalities, genomic instability, increased tumorigenesis	(Serrano, Martinez-Redondo et al. 2013)
Sirt3 -/-	Viable	(Lombard, Alt et al. 2007)
Sirt4 -/-	Viable	(Haigis, Mostoslavsky et al. 2006)
Sirt5 -/-	Viable	(Lombard, Alt et al. 2007)
Sirt6 -/-	Die at PN24, severe hypoglycaemia, lymphopenia, reduced bone marrow, decreased bone mineral density	(Mostoslavsky, Chua et al. 2006)
Sirt7 -/-	Viable, 50% reduced lifespan, premature aging, cardiac hypertrophy	(Vakhrusheva, Braeuer et al. 2008)
p300 -/-	Embryonic lethal GD9.5-11.5, Absent heartbeat, Low contractility, growth retardation, reduced somitogenesis	(Yao, Oh et al. 1998)
p300 +/-	Partial embryonic lethal GD10.5, growth retardation, open neural tube, exencephaly, reduced somitogenesis	(Yao, Oh et al. 1998)
Gcn5 -/-	Embryonic lethal GD10.5, mesodermal layer defects, increased apoptosis, absent neural tube, absent somites	(Xu, Edmondson et al. 2000)
Gcn5 flneo/flneo (Hypomorphic)	Partial embryonic lethality GD18.5, growth retardation, open neural tube, ribs and vertebrae skeletal malformations	(Lin, Zhang et al. 2008, Lin, Zhang et al. 2008)
Ezh2-/-	Embryonic lethal	(O'Carroll, Erhardt et al. 2001)
Ezh2(fl/fl); T:Cre (Mesoderm)	Viable, limb malformation, anteroposterior and proximal-distal defects	(Wyngaarden, Delgado-Olguin et al. 2011)

enzymes during development; less than 45% of expected p300+/- pups survive to weaning (Yao, Oh et al. 1998). Interestingly, several knockdowns exhibit skeletal anomalies; Gcn5-/- embryos die prior to limb bud induction and mesenchymal condensations formation but show defects of the mesodermal layer, suggesting that these processes would be affected (Xu, Edmondson et al. 2000). Indeed, the Gcn5 “flneo” allele encodes for a hypomorphic GCN5 protein with reduced activity. Gcn5 flneo/flneo mice die perinatally and exhibit several defects, including malformation of the ribs and vertebrae (Lin, Zhang et al. 2008, Lin, Zhang et al. 2008). Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of PRC2 that binds the DNA at specific loci and methylates the histone lysine residues in the associated chromatin (Kim, Paylor et al. 2006). Whereas mice carrying a double-allele mutation in this gene do not develop further than the gastrulation stage, conditional knockout mice lacking expression in the mesoderm lineages exhibit abnormal expression of limb bud differentiation markers such as *Tbx5* and *Gli3* as well as limb malformations (O'Carroll, Erhardt et al. 2001, Wyngaarden, Delgado-Olguin et al. 2011).

1.3.2.2 HDACs

Global HDAC1 and HDAC3 knockouts are early embryonic lethal whereas HDAC2 and HDAC8 knockouts die perinatally from severe cardiac malformations and brain haemorrhage, respectively (Montgomery, Davis et al. 2007, Bhaskara, Chyla et al. 2008). Moreover, HDAC8 knockouts also exhibit major growth retardation and skull ossification defects (Haberland, Mokalled et al. 2009). Mice lacking HDAC5 and -9 have enlarged hearts and mice deficient in HDAC4 exhibit major premature ossification

defects, which result in lethality before weaning (Chang, McKinsey et al. 2004, Vega, Matsuda et al. 2004). HDAC7 knockouts die at midgestation from vasculature defects (Chang, Young et al. 2006). Altogether, this suggests many roles for HDACs during development.

1.3.3 HDAC inhibitors (HDACi), sirtuin inhibitor (SIRTi) and cellular signaling

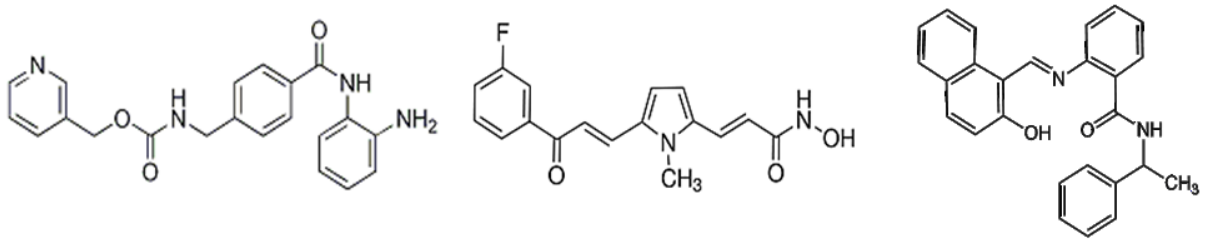
Several inhibitors of the traditional HDACs, classes I, II and IV, have been identified or synthetically developed and characterized (Reviewed in (Xu, Parmigiani et al. 2007)). Although they share the same targets, these compounds have very different structures, chemical families and affinity ranges and can be separated into classes; VPA and sodium butyrate belong to the aliphatic acid group, also called short-chain fatty acid or carboxylates, and both have IC50s in the millimolar (mM) range. Hydroxamic acids include Trichostatin A and Vorinostat, that both have nanomolar affinities for HDACs. Many of the first generation HDACis are pan-inhibitors that inhibit both classes I and II. Recently, class-specific inhibitors have been synthesized and are available (Fig.1.4); MS275 or Entinostat targets specifically HDACs 1, 2 and 3 (Class I), whereas MC1568 targets HDAC4 and 6 (Class II) (Mai, Massa et al. 2005, Beckers, Burkhardt et al. 2007). Several sirtuin inhibitors (SIRTi) such as sirtinol, a SIRT 1/2 inhibitor, have been developed and proposed as treatment for cancer and Parkinson's disease (Reviewed in (Balcerczyk and Pirola 2010)) although none appear to currently be in preclinical trial.

HDACi and SIRTi have effects on several signalling pathways involved in apoptosis, cell cycle arrest and angiogenesis ((Dokmanovic, Clarke et al. 2007, Chiara,

Ilaria et al. 2014)). HDACs are well known for their effects on DNA chromatin remodeling, leading to decreased gene transcription. Yet, exposure to several HDACi has been shown to cause both gene upregulation and downregulation shortly after exposure (Okada, Kushima et al. 2005, Ellis, Pan et al. 2008). Several non-histone proteins that are involved in a number of cellular processes have been shown to interact with HDACs and become acetylated or deacetylated (Glozak, Sengupta et al. 2005, Spange, Wagner et al. 2009). These modifications can affect their activation, protein-protein interactions, stability, as well as nuclear translocation altogether modulating their protein function. .

P53 was the first protein to be identified as a non-histone target of HATs and HDACs (Gu and Roeder 1997); a study by Luo et al. demonstrated that p53 functions were inhibited in cells lacking p300/CBP acetyltransferase activity (Luo, Li et al. 2004). P53 is hyperacetylated following exposure to HDACi and SIRTi in several cancer cell lines (Seo, Jin et al. 2011, Wang, Kim et al. 2012). Since then, other proteins involved in the stress response, apoptosis and DNA repair have been shown to be acetylated. KU70 is involved in the non-homologous end joining repair pathway and also regulates apoptosis by binding and sequestering Bcl-2-associated X protein (BAX) in the cytoplasm. Its acetylation leads to a disruption of protein-protein interaction and the release of BAX (Jeong, Juhn et al. 2007). RelA/Nuclear factor kB (NF-kB) is acetylated by p300 and deacetylated by HDACs and SIRTs although the effects of acetylation on its activity are controversial and seem to be context dependent (Dai, Rahmani et al. 2005, Rothgiesser, Erener et al. 2010). α-Tubulin, a structural protein involved in

Figure 1.4 Chemical structures and IC50s of HDAC class-specific inhibitors, MS275/Entinostat, MC1568 and Sirtinol



MS275 / Entinostat
Class I inhibitor

MC1568
Class II inhibitor

Sirtinol
Class III inhibitor

microtubule formation, is also modified by acetylation and deacetylated by HDAC6 and SIRT2; acetylation at lysine-40 stabilizes the microtubules, structures that are normally highly dynamic (Hubbert, Guardiola et al. 2002, North, Marshall et al. 2003, Al-Bassam and Corbett 2012). Finally, as will be discussed in later sections, SRY-box 9 (SOX9) and Runt-related transcription factor 2 (RUNX2), two skeletal differentiation markers and transcription factors, are regulated by acetylation (Jeon, Lee et al. 2006, Hattori, Coustry et al. 2008).

1.4 Limb long bone formation

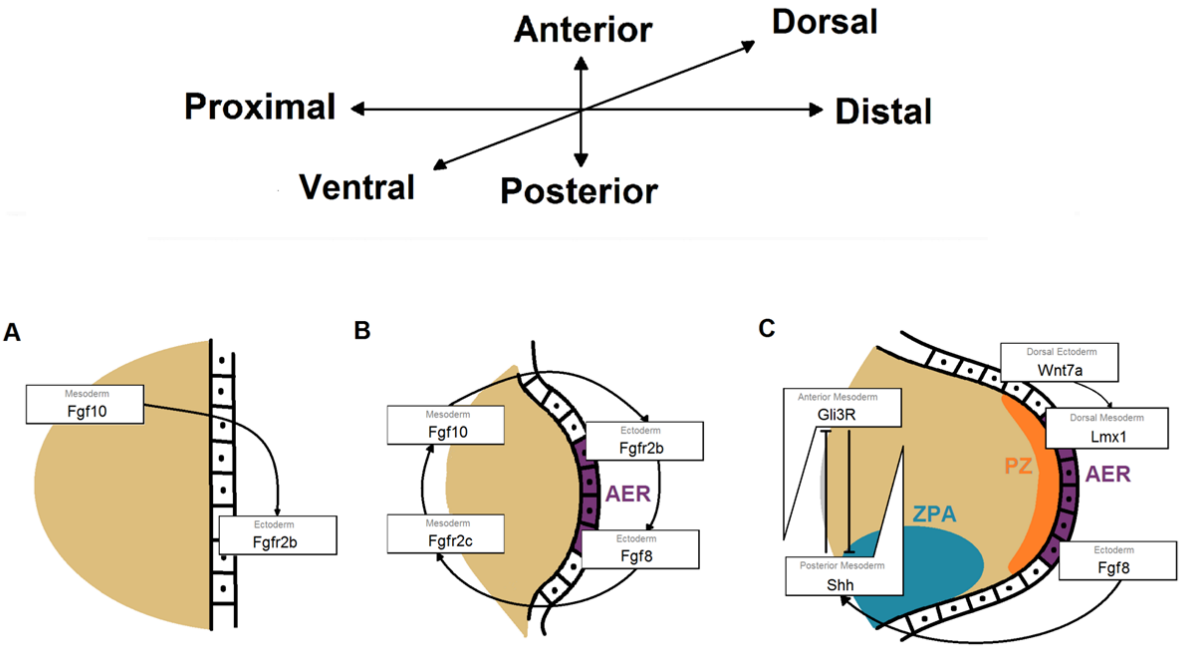
The development of the appendicular anlagen requires the induction of a limb bud in which mesenchymal cells migrate and differentiate, leading to the formation of mesenchymal condensations. During skeletogenesis, these condensations will form a cartilage template that will subsequently be replaced by bone matrix in order to complete the transition into the mature skeleton. The signaling pathways regulating the processes leading to limb development are extremely intricate and much remains unknown. In this introduction, I will provide an overview of the main players involved in this complex network.

1.4.1 Limb bud induction and outgrowth

Limb formation is initiated by the outgrowth of a limb bud from the lateral plate mesoderm at a fixed position along rostral-caudal axis of the embryo. This process is tightly regulated by and completely dependent on FGF10 secretion as the *fgf10* knockout mice completely fail to form these limb buds and suffer from forelimb amelia, a complete absence of forelimb formation. FGF10 secreted from the mesoderm binds to

the FGF receptor (FGFR) 2b in the overlying ectoderm (Fig.5A) (Min, Danilenko et al. 1998, Xu, Weinstein et al. 1998). This leads to the formation of first signaling centre of the limb bud, called the apical ectodermal ridge (AER) that secretes FGF8 and regulates the proximal-distal axis (Fig.1.5B). FGF8 binds the FGFR2c in the underlying mesoderm, called the progress zone (PZ), leading to the release of FGF10, thereby creating a positive feedback loop. Cells in the PZ are actively proliferating and cause the limb bud to grow and expand (Tickle and Eichele 1994). From studies with knockout mice, the WNT/β-catenin and bone morphogenetic protein (BMP) pathways have also been shown to be indispensable for AER formation although the mechanisms regulating their induction are still unclear (Ahn, Mishina et al. 2001, Kawakami, Capdevila et al. 2001). As the outgrowth extends, another signaling centre, called the zone of polarizing activity (ZPA), develops at the posterior side of the limb (Fig.1.5C). This zone secretes sonic-hedgehog protein (SHH) and establishes a SHH-gradient across the anterior-posterior axis that will define patterning (Hornbruch and Wolpert 1991). The most posterior digit, digit 1, does not require SHH to differentiate. This conclusion is further supported by evidence from the *Shh* knock out mice that develop a single digit (Sagai, Hosoya et al. 2005). The proximal-distal and anterior-posterior signaling are closely interconnected; SHH is restricted to the distal-posterior region of the limb by a variety of different factors, including FGF8, a positive regulator, and GLI family zinc finger 3 (GLI3R), a repressor expressed in the anterior region of the limb (Lewandoski, Sun et al. 2000, Wang, Fallon et al. 2000). GLI3 also plays a pivotal role in the regulation of digit as the *Gli3*-/- and the mice expressing SHH anteriorly both exhibit severe pre-axial polydactyly (Sharpe, Lettice et al. 1999, Litingtung, Dahn et al. 2002). The same

Figure 1.5 Overview of key signalling pathways leading to limb bud induction and formation of the limb organizing centre



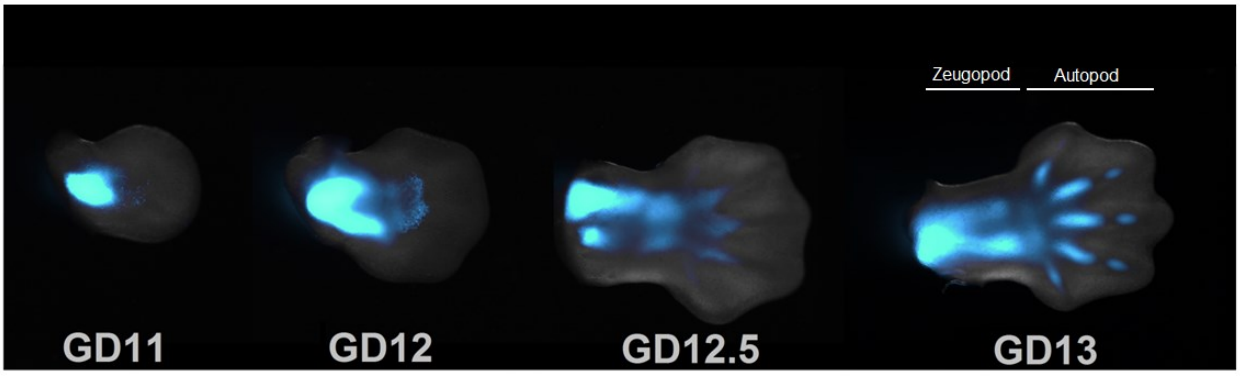
phenotype is seen in human patients with a mutation in the cis-element regulating *Shh* expression (Lettice, Horikoshi et al. 2002). The dorso-ventral axis is defined by its overlaying ectoderm and is largely regulated by the Wnt pathways; WNT7a is solely expressed in the dorsal region of the ectoderm of the developing mouse limb and induces the expression of the LIM homeobox transcription factor 1 (*Lmx1)* gene in the underlying dorsal mesenchyme (Riddle, Ensini et al. 1995) (Parr, Shea et al. 1993). In absence of WNT7a, mice exhibit a ventralisation of their limbs and develop sole pads on both sides of the limb, suggesting that ventral differentiation is the default state and that further induction from ventral factors is not required (Parr and McMahon 1995). Conversely, overexpression of *Lmx1* in the ventral mesenchyme leads to a dorsal patterning (Vogel, Rodriguez et al. 1995). Mutations in these two genes in humans cause Fuhrmann and nail-patella syndromes that share phenotypic similarities with their mouse mutant counterparts (Dreyer, Zhou et al. 1998, Woods, Stricker et al. 2006).

1.4.2 Mesenchymal condensations and endochondral ossification

Skeletogenesis is initiated by the migration and differentiation of mesenchymal cells. They start expressing adhesion molecules such as fibronectin, neural cell adhesion molecule (N-CAM), tenascin and syndecan (Hall and Miyake 1992, Koyama, Leatherman et al. 1995). This leads to their aggregation and the formation of mesenchymal condensations in the future skeletal anlagen (Fig.1.6). From gestational day (GD) 9.5 to 12, the mesenchymal condensations are laid down and three different limb domains are established. The stylopod will later give rise to the humerus, the

Figure 1.6 Formation of mesenchymal condensations in the developing forelimb.

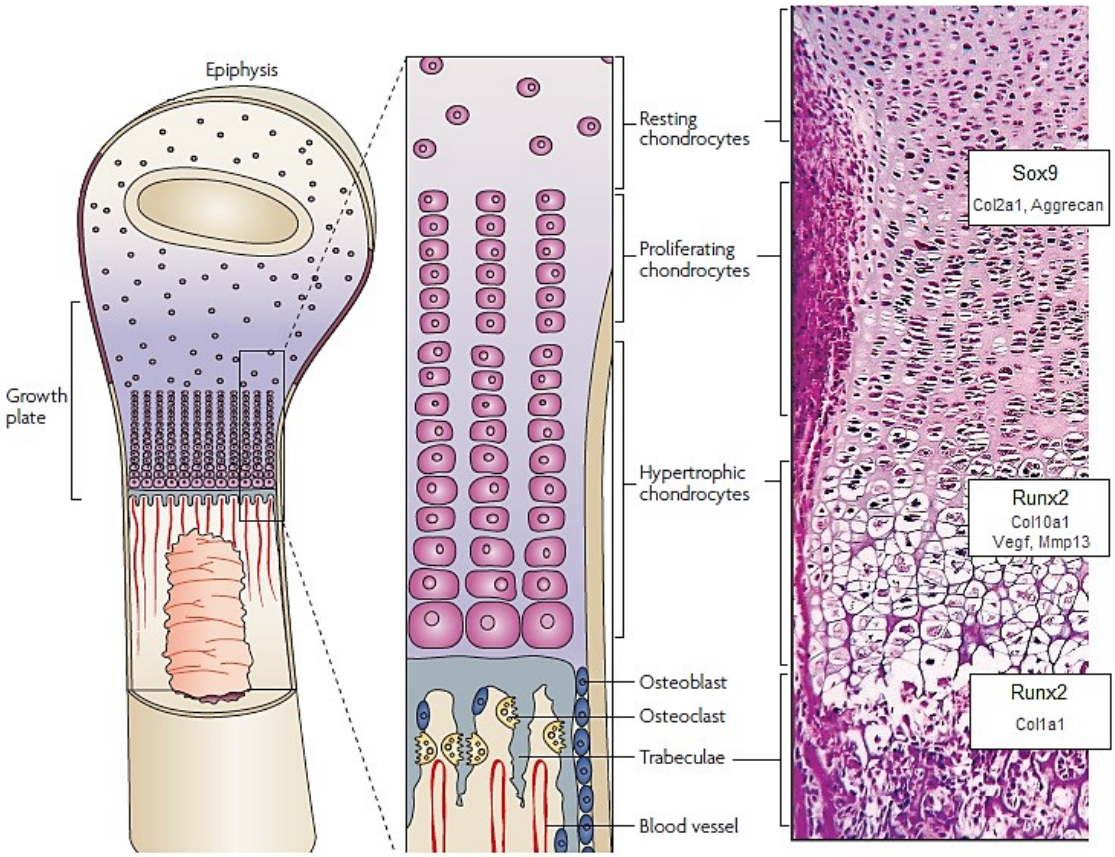
Untreated Col2a1-CFP transgenic embryos were explanted at different timepoints and limbs were photographed using a stereomicroscope. Cyan-fluorescent protein is used as a transgenic marker that identifies the collagen II expressing cells in the developing limb



zeugopod will form the radius and the ulna and the autopod, the hand bones (Saunders 1948). The mesenchymal condensations that are precursors to the flat bones of the body, such as the skull flat bones, scapula and pelvis, are formed through a process called intramembranous ossification and differentiate directly into osteoblasts secreting bone matrix molecules (Franz-Odendaal 2011). The long bones of the body, including the limbs, are formed by endochondral ossification (Fig.1.7)(Kronenberg 2003). This process requires the formation of a cartilaginous template before ossification can occur. In these cases, condensations formed by mesenchymal cells differentiate into non-hypertrophic chondrocytes expressing SOX9 (Lefebvre and Smits 2005). These are further divided into reserve and proliferative chondrocytes. Reserve chondrocytes are small, round and scattered at the border of the growth plate or epiphysis and have a low proliferation rate. Reserve chondrocytes also play a role in the orientation of the growth plate and the inhibition of premature hypertrophy; a current hypothesis is that these cells secrete a morphogen that acts distally to control the alignment and differentiation of proliferative chondrocytes (Abad, Meyers et al. 2002). Proliferative chondrocytes, also called columnar chondrocytes are flattened, tightly stacked in columns parallel to the long axis of the bone and proliferate rapidly as their name suggests. Chondrocytes secrete the extracellular matrix proteins Aggrecan and Collagen type 2 a1 (COL2A1) (Zhao, Eberspaecher et al. 1997, Sekiya, Tsuji et al. 2000). Although SOX9 is the master regulator, a number of factors have been shown to influence the regulation of this pathway. NK3 homeodomain factors 2 (NKX3.2 or BAPX1) is expressed in proliferating chondrocytes and has been shown to regulate and be regulated by SOX9 in a positive feedback loop

Figure 1.7 Cellular organization and signaling molecules in the growth plate during endochondral ossification.

Modified from (Page-McCaw, Ewald et al. 2007) and (Kronenberg 2003)



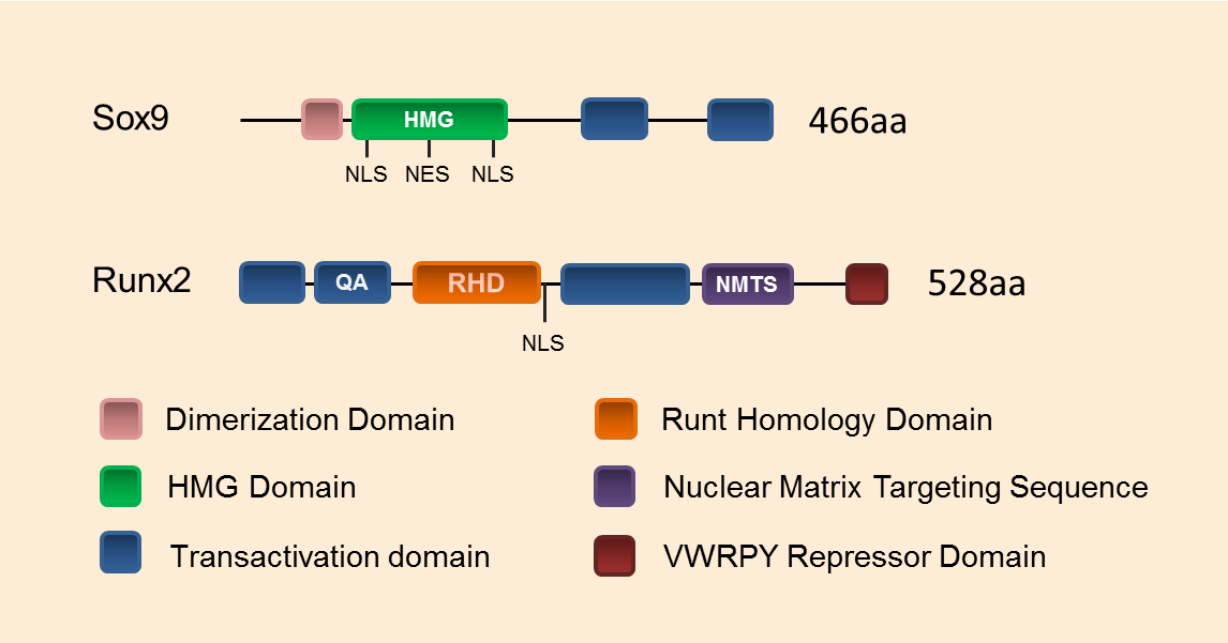
(Zeng, Kempf et al. 2002, Church, Yamaguchi et al. 2005). Moreover, *Nkx3.2* -/- embryos display decreased *col2a1* expression and hypoplastic limbs, suggesting that Nkx3.2 is involved in endochondral ossification. As chondrocytes proceed further from the epiphysis, they cease proliferation and DNA replication and undergo hypertrophy; hypertrophic chondrocytes have a ten times larger cytoplasmic volume than proliferating chondrocytes (Ballock and O'Keefe 2003, Lefebvre and Smits 2005). The main roles of hypertrophic chondrocytes are bone growth and mineralization of the matrix. They stop expressing cartilage markers and express the RUNX2 transcription factor and extracellular matrix proteins such as alkaline phosphatase (ALP) enzyme and collagen type 10 a1 (COL10A1). ALP is a membrane bound enzyme that catalyzes the hydrolysis of phosphate groups necessary for calcification (Poole, Matsui et al. 1989). COL10A1 facilitates the binding of calcium in the extracellular matrix and plays a key role in calcification and mineralization of the bone matrix (Alvarez, Balbin et al. 2001). Hypertrophic chondrocytes also play a pivotal role in attracting vascularization growth factors and matrix degrading enzymes, including members of the matrix metalloproteinase (MPP) family (D'Angelo, Yan et al. 2000). Vascular endothelial growth factor (VEGF) is secreted by hypertrophic chondrocytes (Ballock and O'Keefe 2003). Then, hypertrophic chondrocytes undergo programmed cell death leaving behind a mineralized cartilage-matrix structure filled with cavities where the bone vascular system and primary spongiosa will form (Erenpreisa and Roach 1999, Ballock and O'Keefe 2003). Osteoblasts characterized by the expression of RUNX2 then differentiate within this scaffold and form a Collagen type 1 a1(COL1A1)-rich bone matrix, ultimately creating the trabecular bone (Komori 2010).

1.4.3 SOX9 signaling in the developing limb

SRY-box 9 (SOX9) belongs to the Sox family of transcription factors that share a conserved high-mobility-group (HMG) domain (Fig.1.8) (Kamachi and Kondoh 2013). The HMG domain of SOX9 contains two nuclear localization signals (NLS) and one nuclear export signal (NES) allowing SOX9 to shuttle between the nucleus and the cytoplasm (Smith and Koopman 2004). The HMG domain is also the DNA binding domain. In addition, a dimerization domain can be found near the N-terminus of the protein and two transactivation domains at the C-terminus allow for its interaction with other proteins. Several lysine residues, namely lys61, 253 and 398, have been shown to be acetylated by TIP60, leading to an increase in SOX9 activity (Hattori, Coustry et al. 2008). SOX9 is expressed in a variety of tissues during organogenesis; its tissue-specific expression is regulated, at least partially, by several long-range control elements found upstream and downstream of its promoter (Bagheri-Fam, Barrionuevo et al. 2006). It is found in differentiating mesenchymal cells and chondrocytes and is the master regulator of chondrogenesis. SOX9 directly binds to a consensus binding sequence (A/T)(A/T)CAA(A/T)G in the promoter of *L-Sox5* and *Sox6*, two other members of the HMG-domain containing Sox family, as well as that of *Aggrecan* and *Col2a1* (Harley, Lovell-Badge et al. 1994, Bell, Leung et al. 1997, Han and Lefebvre 2008). Embryonic stem cells lacking both alleles of *Sox9* completely fail to express *Col2a1* and form cartilage matrix even when stimulated with known chondrogenesis-inducing growth factors (Bi, Deng et al. 1999). Mice with a single allele nonsense mutation in *Sox9* exhibit a cleft palate and drastic skeletal anomalies and die perinatally; at birth,

Figure 1.8 Schematic representation of the protein domains of Sox9 and Runx2.

HMG: High-mobility group, NLS: Nuclear localization signal, NES: Nuclear export signal



several bone structures were abnormally bent and hypoplastic (Bi, Huang et al. 2001). Because of the severity of the heterozygote phenotype, it is impossible to obtain complete gene knockouts by traditional methods. However, floxed alleles have been designed for Sox9, allowing conditional knockouts in specific tissues using the Cre/LoxP system (REF). When *Sox9* is removed from the limb bud mesenchyme using paired related homeobox 1 (*Prx1*)-Cre, no limb skeleton is observed although the limb bud outgrowths are formed (Akiyama, Chaboissier et al. 2002). In humans, a single-allele *Sox9* gene mutation results in a condition known as campomelic dysplasia that is often perinatally lethal due to respiratory distress. Those who survive commonly exhibit sex reversal and severe skeletal and craniofacial malformations (Mansour, Offiah et al. 2002).

1.4.4 RUNX2 signaling in the developing limb

Runt-related family of transcription factor (RUNX) genes are also known as the core-binding factor (CBF) α, acute myeloid leukaemia (AML) and polyoma enhancer-binding protein (PEBP) 2α families. Different functions for genes in this family were discovered simultaneously which led to their different names. However, RUNX was designated as the official name by the Nomenclature Committee of the Human Genome Organization (van Wijnen, Stein et al. 2004). Members of this family share a common binding partner CBFβ that does not directly bind the DNA but stabilizes the complex, and a highly conserved Runt domain that is sufficient for binding to the DNA and interaction with CBFβ (Ogawa, Inuzuka et al. 1993, Levanon, Negreanu et al. 1994, Adya, Castilla et al. 2000). A C-terminal VWRPY and proline serine threonine rich (PST) domains are also

common to all RUNX family members. The former is required for interaction with co-repressor complexes whereas the latter acts as a transcription activation domain (Aronson, Fisher et al. 1997, Thirunavukkarasu, Mahajan et al. 1998). A nine amino acid residue nuclear localization signal (NLS) is located between the Runt and PST domains and a nuclear matrix targeting signal (NMTS) is found within the PST domain. The N-terminal domain of RUNX2 is distinct from that of the other members; it contains two activation domains, AD1 and AD2, and a glutamine-alanine domain (QA) (Thirunavukkarasu, Mahajan et al. 1998) (Fig.1.8). Transcription of the RUNX2 gene is regulated by two different promoters; the P1 transcript gives rise to RUNX2-type II, a protein starting with the amino acid sequence MASNS. This isoform is the one most expressed in osteoblasts (Enomoto, Enomoto-Iwamoto et al. 2000, Banerjee, Javed et al. 2001). P2 gives rise to a protein called RUNX2-type I, starting with MRIPV and is expressed in T cells but can also be found in osteoblasts (Harada, Tagashira et al. 1999). However, both isoforms seem to activate the promoters of their target genes, suggesting that the alternative splicing affects their tissue-specific expression but not function (Harada, Tagashira et al. 1999). RUNX2 is regulated at the post-translational level as well and is modified by phosphorylation and acetylation leading to changes in activity (Wee, Huang et al. 2002, Jeon, Lee et al. 2006)

Although members of the RUNX family share many common features, the analysis of the knockouts reveals very distinct roles during organogenesis and development; both the *Runx1*-/- and *Cbfβ*-/- knockouts are embryonic lethal at GD 11.5-13.5 due to a major defect in hematopoiesis (Sasaki, Yagi et al. 1996, Wang, Stacy et al. 1996). Mice lacking *Runx3* are viable but exhibit gastric epithelium hyperplasia as well as dorsal root

neuron anomalies (Levanon, Bettoun et al. 2002, Li, Ito et al. 2002). In contrast, Runt-related transcription factor 2 (RUNX2/CBFα1/AML3/PEBP2αA) has a key role in chondrocyte differentiation and osteogenesis.

Although *Runx2* knockout mice survive through embryogenesis, they die shortly after birth from respiratory failure because of the complete absence of ossified skeleton (Otto, Thornell et al. 1997). In these mice, both intramembranous and endochondral ossification is blocked; without Runx2, osteoblast progenitors fail to differentiate into mature osteoblasts and to express the extracellular matrix proteins required for bone formation (Komori, Yagi et al. 1997). In humans, several mutations in *Runx2* can lead to a condition called cleidocranial dysplasia (CCD), characterized by severely underdeveloped clavicles, narrow shoulders and spine scoliosis. Moreover, these patients have lower bone density and increased risk of developing osteoporosis (Quack, Vonderstrass et al. 1999, Zhang, Yasui et al. 2000). The prevalence of CCD is about 1 per million individuals worldwide (Mundlos, Mulliken et al. 1995).

Runx2 binds directly to the DNA via its Runt homology domain at a conserved nucleotide binding sequence (Pu/T)ACPuCA, where Pu indicates purines (Ducy, Zhang et al. 1997). Runx2 directly binds the promoter of *osteocalcin, osteopontin and col1a1* in vitro (Ducy, Zhang et al. 1997). Overexpression of Runx2 in different cell lines causes an upregulation of MMP13, ALP, VEGF and COL10A1 (Enomoto, Enomoto-Iwamoto et al. 2000, Zelzer, Glotzer et al. 2001).

1.5 Programmed cell death

Programmed cell death, also known as apoptosis, is a conserved process among organisms to remove unwanted or sick cells while causing minimal damage to the surrounding cells. It is characterized by a series of hallmarks such as the translocation of phosphatidylserine to the outer plasma membrane, the activation of cysteine-asparagine proteases called caspases, condensation and fragmentation of the nucleus (Kerr, Wyllie et al. 1972, Martin, Reutelingsperger et al. 1995). Although both caspase-dependant and -independent programmed cell death have been described, we will focus on the former and provide a brief overview of molecular pathways involved in its regulation and role during development.

1.5.1 Apoptotic pathways

Apoptotic caspases are separated into the initiator class (Caspase 2, 8, 9 and 10) and the effector caspases (Caspase 3, 6 and 7). Although they are subject to both transcriptional and posttranslational regulation, caspases are constantly expressed and their inactive form is present in the cytosol. Their activation is regulated at the post-translational level by the protease-mediated cleavage of the protein at an asparagine residue. The caspase-dependent apoptotic response is triggered by extrinsic or intrinsic pathways (Fig.1.9); the former is induced by the binding of FasL, an extracellular ligand, to the death receptor Fas, leading to the formation of a cytosolic complex constituted of fas-associated protein with death domain (FADD) and Caspase 8. Caspase 8 auto-cleaves and activates itself leading to the proteolytic activation of its downstream target, Caspase 3, an effector caspase previously known as CPP32 (Nagata 1999). The

Figure 1.9 Schematic overview of the caspase-dependent extrinsic and intrinsic apoptotic pathways.

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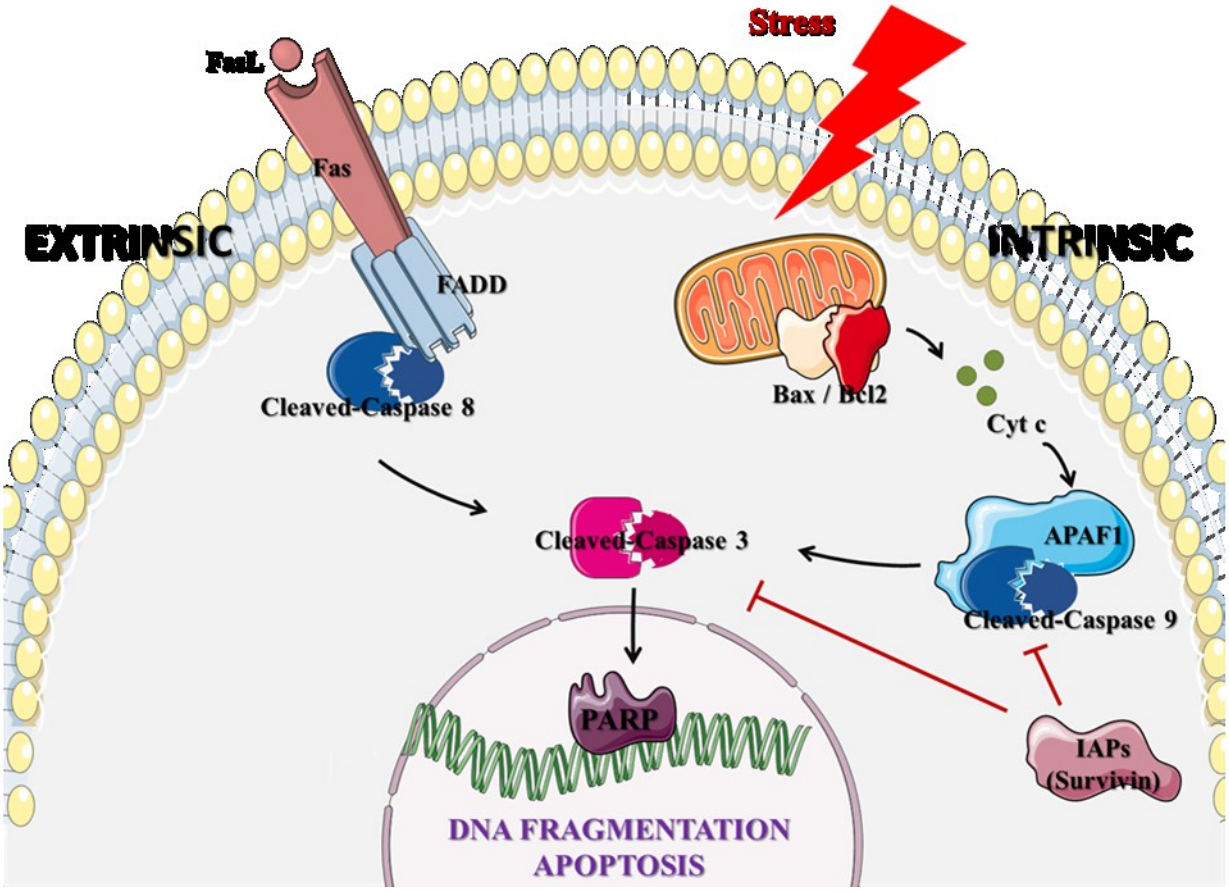


intrinsic pathway is mediated by mitochondrial factors, the B-Cell Lymphoma 2 (BCL2) family of proteins. BCL2, BAX and Bcl-2 homologous antagonist/killer (BAK) are anti-apoptotic and pro-apoptotic members of this family that regulate mitochondrial membrane pore opening. When they are dysregulated, an opening is created in the mitochondrial membrane, allowing the release of proapoptotic factors such as cytochrome c. Cytochrome c then binds to the apoptotic-protease-activating-factor 1 (APAF1), thereby inducing a conformational change and activating the complex. This complex, called the apoptosome, mediates the activation of caspase 9; activated caspase 9 will subsequently cleave caspase 3 (Zou, Li et al. 1999). Activated caspase 3 cleaves the death substrate, poly-ADP-ribose polymerase (PARP), that plays important roles in DNA strand break repairs and chromosome stability (Tewari, Quan et al. 1995, Wang, Stingl et al. 1997). The cleavage of caspase 3 is a central point to both the extrinsic and intrinsic pathways and was shown to be essential for nuclear apoptosis and DNA fragmentation (Hirata, Takahashi et al. 1998). In contrast, a family of proteins called the inhibitors of apoptosis protein (IAP) provides an additional layer of caspase regulation; they contain at least one BIR domain that mediates protein-protein direct interactions with caspases. Survivin/BIRC5 is a member of this family and directly inhibits caspase 3 (Tamm, Wang et al. 1998, Altieri 2010).

1.5.2 P53 signalling

P53 is a very well established transcription factor and tumor suppressor that plays a pivotal role in cellular response to different stresses. It regulates the transcription of

genes involved in cell-cycle arrest, metabolism, apoptosis and DNA repair (Harms, Nozell et al. 2004, Vousden and Ryan 2009).

Its molecular structure contains an N-terminal TAD, a DNA-binding domain, and a C-terminal domain containing a tetradimerization domain and both a NLS and a NES that allow its nuclear-cytoplasmic shuttling (Shaulsky, Goldfinger et al. 1990, Stommel, Marchenko et al. 1999, Toledo and Wahl 2006). Under normal conditions, p53 is kept at a low level in the cytoplasm through the regulation of the ubiquitin ligase mouse double minute 2 homolog (MDM2) (Lavin and Gueven 2006). MDM2 binds to the TAD of p53 and causes lysine residues in its C-terminal domain to become ubiquitinated, thereby targeting it for degradation. Under stress conditions kinases, such as checkpoint kinase 2 (CHK2), phosphorylate serine/threonine residues in the TAD. This disrupts the interaction with MDM2 and stabilizes p53. Also, several lysine residues in the C-terminal domain of p53 have been shown to be acetylated; this both increases the DNA-binding activity of the protein and competes with ubiquitination (Nakamura, Roth et al. 2000, Ito, Lai et al. 2001). P53 directly binds the promoter region of target genes at the consensus binding sequence of two repeats of a decamer motif PuPuPuC(A/T)(A/T)GPyPyPy, where Pu and Py are purines and pyrimidines, respectively (el-Deiry, Kern et al. 1992). P53 is a transcription factor that acts both as a repressor and activator of transcription of a long list of different targets via a series of mechanisms (Reviewed in (Rinn and Huarte 2011)). It non-competitively binds the promoter region of *Survivin* and *Bcl2* and inhibits the transcriptional activators of those genes, thereby leading to their repression and a pro-apoptotic effect (Budhram-Mahadeo, Morris et al. 1999, Hoffman, Biade et al. 2002). It downregulates several cyclins and cyclin kinases, such as cyclin A2, cyclin B1

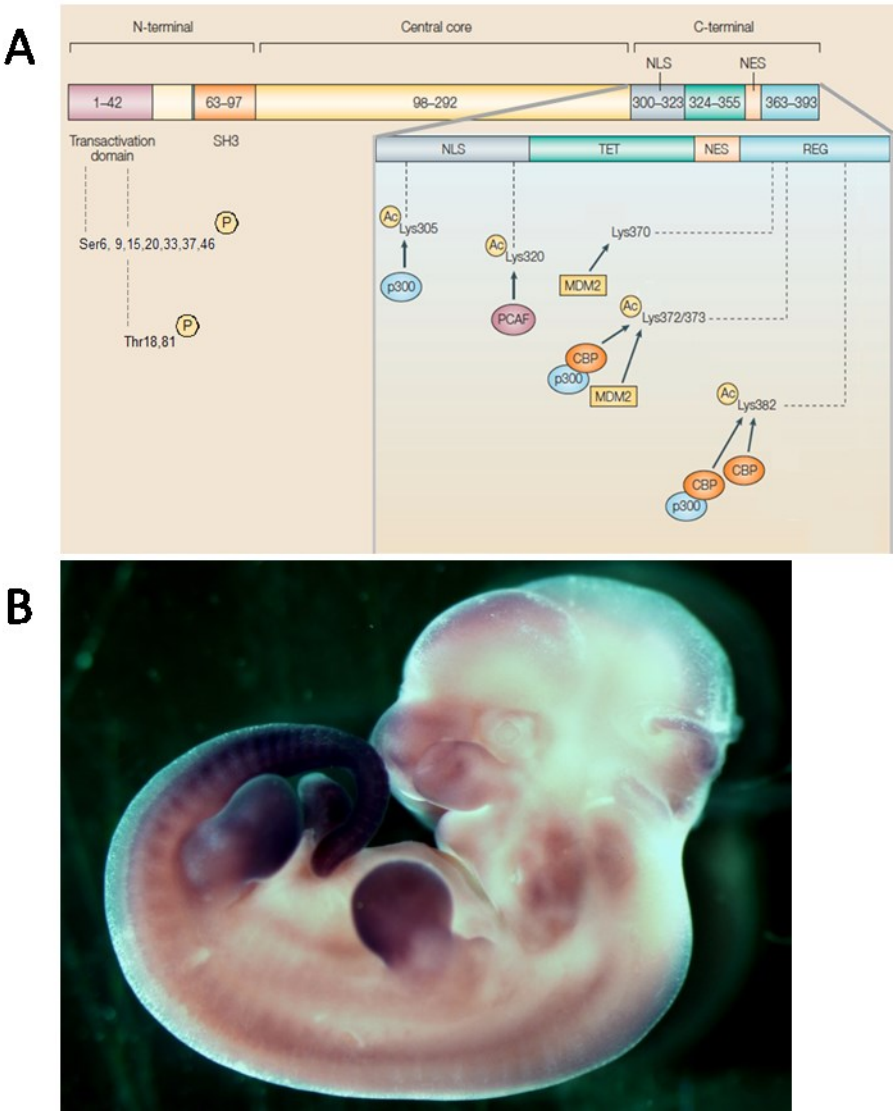
and cyclin-dependent kinase 1 (CDK1), and upregulates cell cycle repressors, such as cyclin-dependent kinase inhibitor 1 (Cdk1/waf1/cip1/p21), leading to its effect on cell cycle arrest (el-Deiry, Harper et al. 1994, Di Agostino, Strano et al. 2006). Loss-of-function mutations in the p53 gene both in humans and in animal models suggest that it is not required for embryonic development; *p53*-/- mice are viable at birth although they develop tumors as early as 3 to 6 months of age (Tsukada, Tomooka et al. 1993). In humans, mutations in the *p53* gene result in a condition called familial Li-Fraumeni syndrome, characterised by the early onset of diverse cancers, including sarcomas, breast cancers and brain tumors, but not an increased risk of birth defects (Birch, Alston et al. 2001). In contrast, mice lacking MDM2, the inhibitor of p53, die early during embryogenesis (GD 4.5-7.5), whereas *p53-mdm2* double knockouts are viable and have a normal phenotype at birth (Jones, Roe et al. 1995). In mid-organogenesis stage embryos, p53 is highly expressed in the limb buds yet little is known about its action in limb organogenesis (Fig.1.10) (EMAGE gene expression database (http://www.emouseatlas.org/emage/, (Richardson, Venkataraman et al. 2014).

1.5.3 Cell death in organogenesis and limb formation

Cell death plays a crucial role in embryonic development and organ formation and in some cases, is detected as rapidly as at the two-cell stage of a mammalian embryo although its role at that stage remains to be deciphered (Penaloza, Lin et al. 2006). Subsequently, cell death is required for heart, inner ear, and skeletal muscle formation (Abdelwahid, Pelliniemi et al. 2002, Avallone, Balsamo et al. 2002, de Torres, Munell et al. 2002). It plays a role in the central nervous system; 20 to 80% of neurons that initially

Figure 1.10 P53 protein structure and expression during organogenesis.

A. Schematic representation of the protein domains and posttranslational modification sites of p53. Modified from (Bode and Dong 2004). B. In situ hybridization of p53 mRNA expression in a mid-organogenesis mouse embryo. Modified from (Richardson, Venkataraman et al. 2014)



differentiate during embryogenesis undergo programmed cell death (Gordon 1995). *Caspase3* knockout mice exhibit increased embryonic and perinatal lethality and the survivors exhibit a variety of defects that include hydrocephaly and decreased apoptosis in the cortex and cerebellum, resulting in a higher number of neurons (Kuida, Zheng et al. 1996). Caspase mediated apoptosis also plays an important role during osteogenesis as it is required for the removal of hypertrophic chondrocytes in the growth plate, as described above. *Caspase3* -/- and +/- mice exhibit delayed ossification, decreased mineral density and shorter bone length (Miura, Chen et al. 2004). Mice lacking both *Bax* and *Bak* generally die perinatally and exhibit persistent interdigital webbing, also called syndactyly (Lindsten, Ross et al. 2000).

Although the complete mechanism underlying drug-induced teratogenicity remains unknown, several studies have established a correlation between an increase in cellular apoptosis in target organs and teratogenicity; teratogens such as ethanol, retinoic acid, and cyclophosphamide, an anticancer drug, as well as many others, induce apoptosis in the developing fetus (Cartwright and Smith 1995, Huang and Hales 2002, Ali-Khan and Hales 2003). Altogether, this suggests that well regulated apoptosis is required during embryonic development and both a reduction and excess cell death can lead to abnormal differentiation.

1.6 Rationale

Although folic acid supplementation has caused an overall drastic decrease in the congenital anomaly rate (~ 1%) over the past few years, 3-5% of the live birth or stillbirths in Canada annually have a serious congenital anomaly; 25% of stillborns

deaths are accompanied or due to a major congenital malformation (PHAC 2013). Three to eight infants per 10000 (approximately 100 to 300 per year) are affected with a limb deficiency defect and at least 30% of these infants exhibit another congenital malformation (PHAC 2013). Although it has been known for decades that in utero exposure to some drugs and environmental toxicants increases the risk of birth defects, the mechanism of action of these teratogens still remains unresolved. VPA is a widely used and studied medication and its teratogenicity has been well described in both humans and animal models. Therefore, it is used as a model teratogen to investigate the molecular pathways leading to adverse outcomes. Our overall goal is to further our understanding of these mechanisms in order to establish prevention strategies and facilitate the identification of new potential developmental toxicants.

1.7 Hypothesis and objectives

Limb pattern formation involves several well characterized signalling pathways that drive cellular processes, including chondrocyte differentiation, bone mineralization and interdigital apoptosis. I hypothesize that VPA causes limb malformations by inhibiting HDACs, leading to altered regulation of cell differentiation and death during limb development.

1.7.1 Objective I

To examine the effects of VPA and its analog, valpromide (VPD) on limb morphological differentiation, chondrogenesis and osteogenesis and to identify the gene(s) mediating these effects.

1.7.2 Objective II

To investigate how VPA and VPD affect p53 signaling and caspase-dependant cellular apoptosis in the limb.

1.7.3 Objective III

To decipher the effect of individual HDAC classes on limb chondrogenesis, osteogenesis and cellular apoptosis using pharmacological HDAC class-specific inhibitors

Chapter 2 Exposure to Valproic Acid Inhibits Chondrogenesis and Osteogenesis in Mid-Organogenesis Mouse Limbs

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Abstract

In utero exposure to valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, causes neural tube, heart and limb defects. Valpromide (VPD), the amide derivative of VPA, does not inhibit HDAC activity and is a weak teratogen in vivo. The detailed mechanism of action of VPA as a teratogen is not known. The goal of these studies was to test the hypothesis that VPA disrupts regulation of the expression of genes that are critical in chondrogenesis and osteogenesis during limb development. Murine gestation day 12 embryonic forelimbs were excised and exposed to VPA or VPD in a limb bud culture system. VPA caused a significant concentration-dependent increase in limb abnormalities that was correlated with its HDAC inhibitory effect. The signaling of both *Sox9* and *Runx2*, key regulators of chondrogenesis, was downregulated by VPA. In contrast, VPD had little effect on limb morphology and no significant effect on HDAC activity or the expression of marker genes. Thus, VPA exposure dysregulated the expression of target genes directly involved in chondrogenesis and osteogenesis in the developing limb. Disturbances in these signaling pathways are likely to be a consequence of HDAC inhibition since VPD did not affect their expression.

Keywords: teratogen; histone deacetylase inhibitor; *Sox9*; *Runx2*

2.1 Introduction

Valproic acid (VPA) is commonly used to treat epilepsy and bipolar disorders; moreover, it is currently being tested as an anticancer agent (Duenas-Gonzalez et al., 2008). VPA is also a teratogen; in utero exposures may result in the induction of neural tube defects, heart abnormalities, craniosynostosis and skeletal malformations, such as ectrodactyly and syndactyly (Sharony, Garber et al. 1993, Lajeunie, Barcik et al. 2001, Vajda and Eadie 2005, Ornoy 2006). Although a number of mechanisms have been proposed for the anticonvulsant and antidepressant actions of VPA (reviewed in (Terbach and Williams 2009)), the molecular signaling pathways by which VPA exposures lead to malformations is not known. VPA is an inhibitor of class I and II histone deacetylases (HDACs) (Phiel, Zhang et al. 2001). Furthermore, recent studies have suggested that the teratogenicity of several compounds is linked to their ability to inhibit HDACs; apicidin, MS-275, sodium butyrate and sodium salicylate are all HDAC inhibitors that induce defects of the axial skeleton in mice (Di Renzo, Broccia et al. 2007, Di Renzo, Cappelletti et al. 2008). Valpromide (VPD), a structural analog of VPA, has been reported to have little potency, either as an HDAC inhibitor or as a teratogen (Phiel, Zhang et al. 2001, Okada, Aoki et al. 2004). The mechanisms by which HDAC inhibitors interfere with bone development remain unknown.

The long bones of the limb are formed by endochondral ossification (reviewed in (Kawakami, Rodriguez-Leon et al. 2006). In the first step, chondrogenesis leads to the formation of a cartilage template that is subsequently replaced in the second step by bone mineralization, during the osteogenesis phase. Chondrogenesis is a very tightly

regulated process during which chondrocytes undergo sequential proliferation, differentiation and hypertrophy. Each of these steps is characterized by the expression of specific marker genes. Sex-determining-region Y (SRY)-box 9 (*Sox9*) and its two downstream target genes, *L-Sox5* and *Sox6*, are expressed in early proliferative chondrocytes and form a complex that regulates the transcription of type II collagen (Col2a1) and Aggrecan, two proteins involved in extracellular matrix formation. *Sox9* heterozygote mice exhibit limb malformations; conditional *Sox9* knockout in the limb mesenchyme results in a complete failure to form any cartilage, clearly illustrating the pivotal role this gene plays in chondrogenesis (Bi, Huang et al. 2001, Akiyama, Chaboissier et al. 2002). Once the proliferative stage is terminated, the chondrocytes undergo hypertrophy and begin to express Runt-related factor 2 (*Runx2*), a key regulator of hypertrophic differentiation, that induces the transcription of collagen 10a1 and the mineralization of the surrounding matrix (Ding, Lu et al. 2012). In a recent review, Bradley et al. highlighted the importance of the different roles of HDACs in both endochondral and intramembranous ossification (Bradley, McGee-Lawrence et al. 2011). However, little is known about the effects of VPA, or any other known teratogen, on the regulation of chondrogenesis in the developing limb.

In this study, we tested the hypothesis that the inhibition of HDAC activity by valproic acid disturbs the expression of key genes that regulate chondrogenesis and osteogenesis, leading to limb malformations. To elucidate how VPA affects these processes, we exposed mouse embryonic limb buds to increasing concentrations of VPA and its analog, VPD, in an *in vitro* limb bud culture system. We then analyzed the effects of these compounds on histone acetylation, the morphology and differentiation of

limb cartilage, and the expression of genes involved in limb bone formation. Our results show that VPA induces histone hyperacetylation; this epigenetic modification is directly correlated with inhibition of the expression of markers of both chondrogenesis and osteogenesis at early time points after exposure and with the subsequent limb development defects that are observed.

2.2 Materials and Methods

Limb bud cultures and drug treatments

Time-pregnant CD1 mice (20-25 g) purchased from Charles River Canada Inc. (St-Constant, QC, Canada) were euthanized by cervical dislocation on gestation day 12 and their embryos were explanted. All animal studies complied with the guidelines established by the Canadian Council on Animal Care under protocol 1825. The embryonic forelimbs were cultured as previously described (Huang and Hales, 2002). Briefly, limbs were excised in Hank's balanced salt solution, pooled and cultured *in vitro* in 6 ml culture medium consisting of 75% BGJb medium (GIBCO BRL Products, Burlington, ON, Canada), 25% salt solution supplemented with ascorbic acid (160 µg/ml), and gentamycin (1 µl/ml, GIBCO BRL Products). Each culture was gassed with 50% O_2, 5% CO_2 and 45% N_2. Different concentrations of sodium valproate (VPA, Sigma, St-Louis, MO, #P4543), dissolved in distilled water, or valpromide (VPD, Katwijk Chemie, Netherlands), dissolved in DMSO, were added to designated cultures. No differences were observed between limbs cultured with DMSO or water (data not shown).

Limb morphology

Forelimbs were cultured for 6 days at 37°C with a change of medium and re-oxygenation on day 3; VPA was not added to the culture medium at that time. Limbs were then fixed overnight in Bouin's fixative, stained with 0.1% toluidine blue (Fisher Scientific, Nepean, ON, Canada) in 70% ethanol for 24h, dehydrated using a gradient of ethanol and stored in cedarwood oil (Fisher Scientific). Limbs were observed using a dissection microscope and the morphology and differentiation of each limb was assessed using a limb morphogenetic differentiation scoring system (Neubert and Barrach, 1977). Briefly, this system attributes a score to the radius, the ulna, the carpalia, and each one of the 5 digits according to their differentiation. Five separate replicates (n = 5 bottles for each treatment and time, with 7-10 limbs per bottle) were *done.*

Real-Time qRT-PCR

Total RNA from homogenized limbs (4-5 limbs per group) was extracted using an RNeasy Microkit (Qiagen, Mississauga, ON, Canada). The RNA concentration and purity of each sample were assessed by spectrophotometry using a Nanodrop1000 spectrophotometer (Fisher Scientific). The samples were diluted to a working concentration of 10 ng/µl and transcripts were quantified using Quantitect One-Step SYBR Green RT-PCR (Qiagen) and the Roche LightCycler. The primer sets were designed using Primer 3 software (Table 1) and produced by alpha DNA (Montreal, QC, Canada) with the exception of the *Runx2* primers that were purchased from Qiagen

Table 2.1 Primer sequences used for quantitative RT-PCR

Gene	Sequence	
Sox9	Forward:	GATGCAGTGAGGAGCACTGA
	Reverse:	TATCCACGGCACACACACTT
Sox5	Forward:	AAGCAGCAACAGGAGCAGA
	Reverse:	AAACCAGGAGGAAGGAGGAA
Sox6	Forward:	AACGACCACACCATCACCTC
	Reverse:	AGGCTTCCATTTCATCCTCTC
Col2a1	Forward:	GAAGGTGCTCAAGGTTCTCG
	Reverse:	CTTTGGCTCCAGGAATACCA
Col10a1	Forward:	ACCCCAAGGACCTAAAGGAA
	Reverse:	CCCCAGGATACCCTGTTTTT
18s rRNA	Forward:	AAACGGCTACCACATCCAAG
	Reverse:	CCTCCAATGGATCCTCGTTA

(#QT00102193). The reaction was done in a final volume of 20 µl that was composed of 10 µl SYBR Green Master Mix, 2 µl of each forward and reverse primer in a 10 µM solution, 0.2 µl Quantitect Reverse Transcriptase mix, 4.8 µl RNAse-DNAse-free water and 1 µl sample. PCR was done under the following conditions: 20 min at 50°C followed by 50 cycles of 95°C for 15 min, 94°C for 15 s, 55°C for 30 s, and 72°C for 20s. Serial dilutions of non-treated hindlimb RNA samples were used to create a standard curve. Each reaction was done in duplicate, averaged and normalized to the amount of 18S RNA transcripts. Each experiment was replicated 6-12 times per group.

Western blotting

Cultures were ended at specified times. Limbs were homogenized by sonication in lysis buffer containing protease inhibitors: 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5, 40 µg/ml bestatin, 0.2 M phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 6 µg/ml aprotinin. Total protein was extracted and quantified using spectrophotometric Bio-Rad protein assays (Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins (15-20 µg per sample) were separated by SDS-PAGE acrylamide gel electrophoresis and transferred on polyvinylidene difluoride membranes (BioSciences Inc., Baie d'Urfé, QC, Canada). Precision standards (Bio-Rad Laboratories) were used as molecular weight markers. Membranes were blocked in 5% non-fat milk in TBS-T (137 mM NaC1, 20 mM Tris pH 7.4, 0.05% Tween 20) for 1h at room temperature, probed overnight at 4°C with primary antibodies, washed and incubated with the secondary antibody for 2h at room temperature. Immunoblotting was done using polyclonal antibodies against Histone 4

lysine 12 acetylation (H4K12ac, EMD Millipore, Billerica, MA, 1:5000), SOX9 (Abcam, Cambridge, MA, 1:500) and beta-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:10000). The secondary antibodies, conjugated to horseradish peroxidase (HRP), were donkey anti-rabbit antibodies (1:5000, GE Healthcare Limited, Baie d'Urfé, QC, Canada) for H4K12ac and SOX9 and anti-goat antibodies (Santa Cruz Biotechnology, Inc, 1:10000) for beta-actin. Western blots were visualized with the Enhanced Chemiluminescence Plus Kit (GE Healthcare Limited) and quantified by densitometry using a Chemi-Imager 400 imaging system (Alpha Innotech, San Leandro, CA).

Primary cell cultures

Embryonic forelimbs were collected on gestation day 12 and washed with low calcium Hank's balanced salt solution (HBSS) (Hank's Balanced Salt Solution Ca/Mg free [Gibco, Life Technologies, Burlington, ON], 1 M Hepes pH 7.4, 0.15 M $CaCl_2$). Limbs were cut and incubated in a collagenase solution (1.5mg/mL collagenase type II, 300mg/mL bovine serum albumin in low Ca HBSS) for 2h at 36°C. Cells were sedimented from the suspension by centrifugation at 1000 x g for 5 min and incubated with 2.5 g/mL Pancreatin for 15 min at room temperature. Cells were then washed with complete medium 199 (Medium 199, GIBCO, Life Technologies, 26.2 mM $NaHCO_3$, 25 mM Hepes pH7.4, 0.06 mg/mL gentamicin sulfate, 1% 100X Antibiotic-Antimycotic, 10% fetal bovine serum) and passed through a 40µm filter cloth. Cells were counted with a hemocytometer and approximately 1 x 10^6 cells were plated in a 12 well culture plate for 24h. Cells were then treated with vehicle or VPA (3.6 mM) with or without 5 µg/mL

actinomycin D (Sigma). Cultures were stopped at designated times and total RNA was extracted and analyzed by qRT-PCR. The experiment was repeated 9 times.

Alcian Blue staining

Limbs were cultured for 24h and fixed overnight in 95% ethanol. Limbs were then transferred to alcian blue solution (15 mg alcian blue, 80 ml 95% ethanol, 20 mL glacial acetic acid) for 24h at room temperature and again to 95% ethanol for 24h. Limbs were transferred to 1 part 95% ethanol: 1 part glycerol solution and visualized by light microscopy; staining was qualitatively assessed. The experiment was repeated 5 times with 7-10 limbs/concentration and time in each experiment.

Statistical Analyses

All morphology and gene expression data sets were analyzed statistically using Systat 10.2 (Systat Software, Point Richmond, CA). The Mann-Whitney U test with Bonferonni's multiple-comparison correction was used to compare all concentration groups within a given time point. The minimum level of significance was $P < 0.05$.

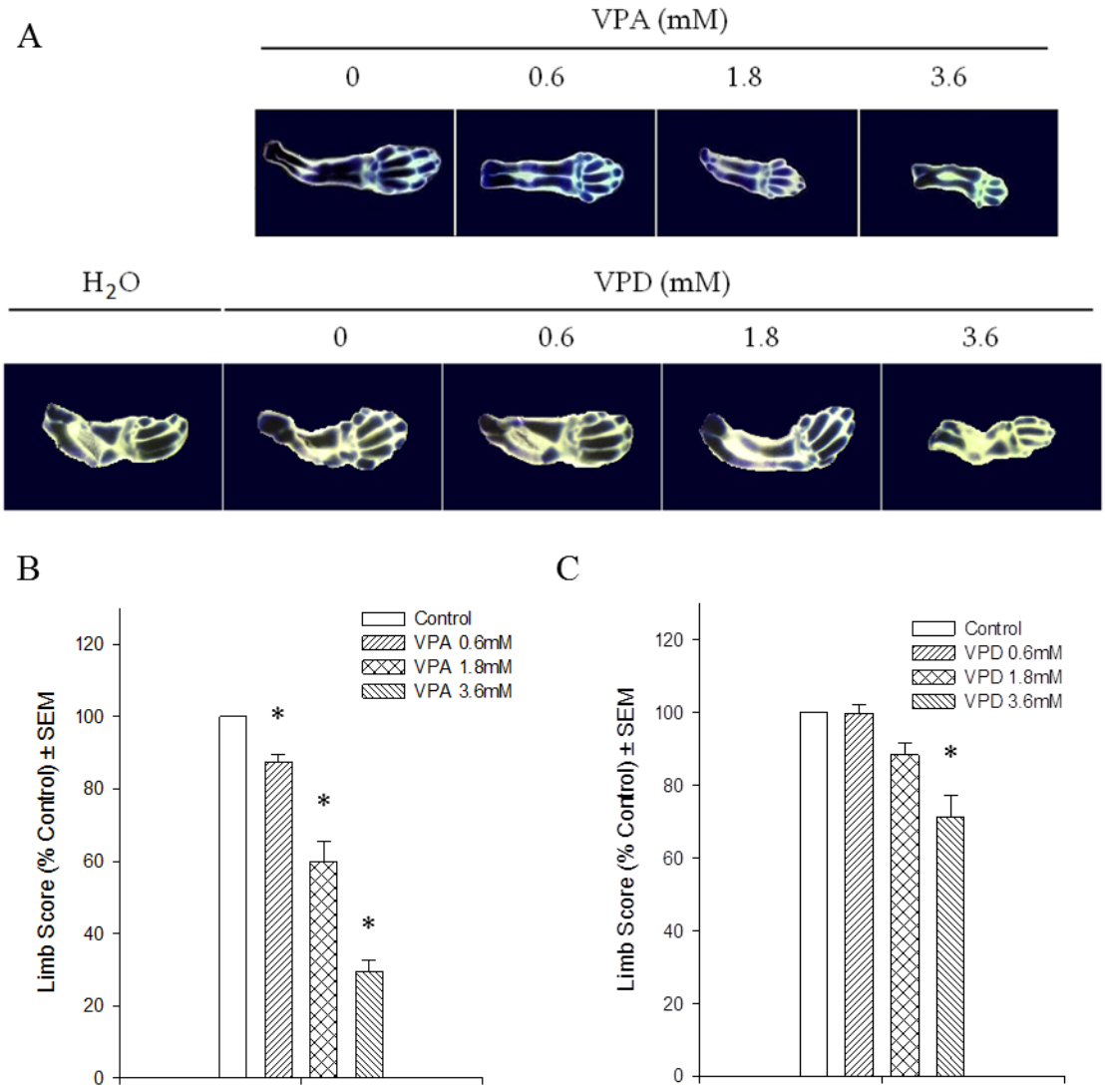
2.3 Results

Effects of VPA and VPD on limb morphology

The effects of VPA on the morphology and differentiation of embryonic forelimbs cultured in vitro are shown in Figure 2.1A. The limbs gross appearance is slightly

Figure 2.1 Limb morphology following exposure to VPA or VPD

Embryonic day 12 forelimbs were cultured in the presence of increasing concentrations of VPA or VPD (0, 0.6, 1.8 or 3.6 mM) for 6 days. They were stained with 0.1% toluidine blue (A) to visualize cartilage formation and scored according to their morphology (B-C). * = $p<0.05$.



variable and some appear to be distorted due to the rotating culture system. However, the growth and differentiation of the control limbs were all normal; Cartilage template was properly formed and long elongated digits with phalanges were observed. Although the low dose VPA treatment group showed minimal effects on morphology, the limbs exposed to 1.8 or 3.6 mM VPA exhibited a marked decrease in growth and differentiation. The long bones were reduced in size, the staining of the carpalia was decreased and the metacarpals were short and thick. Phalanges were often missing and, in the highest concentration group, several limbs with oligodactyly were observed. The morphology and state of differentiation of the limbs was assessed quantitatively using a limb morphogenetic differentiation scoring system (Figure 2.1B). A significant concentration dependent decrease in the limb score was observed in all VPA treatment groups. These results showed that VPA inhibited the development of the limbs *in vitro* and caused a pronounced decrease in cartilage formation. In contrast, limbs exposed to 0.6 mM or 1.8 mM VPD were not different from control limbs (Figure 2.1A). Limbs exposed to 3.6 mM VPD exhibited a mild phenotype of under development of the phalanges; this resulted in a small but significant reduction in limb score (Figure 2.1C).

Effects of VPA and VPD on histone acetylation

To determine if the phenotype observed following VPA exposure was associated with HDAC inhibition, we examined the level of histone-4 acetylation as an indicator of global HDAC activity. VPA caused a rapid and concentration-dependent hyperacetylation of histone 4, as early as 1h following exposure; this was maintained up to 6h (Figure 2.2A). VPD did not cause detectable changes in histone 4 acetylation (Figure 2.2B).

Figure 2.2 VPA induction of histone 4 lysine 12 acetylation.

H4K12 acetylation was used as a marker of HDAC inhibition and normalized to -Actin. Western blot of whole cell protein extracts from VPA-treated (A) and VPD-treated (B) limbs at 3 hours following exposure (Left). Limbs were cultured for 1, 3 or 6h and proteins were quantified by densitometry (Right). * = p<0.05

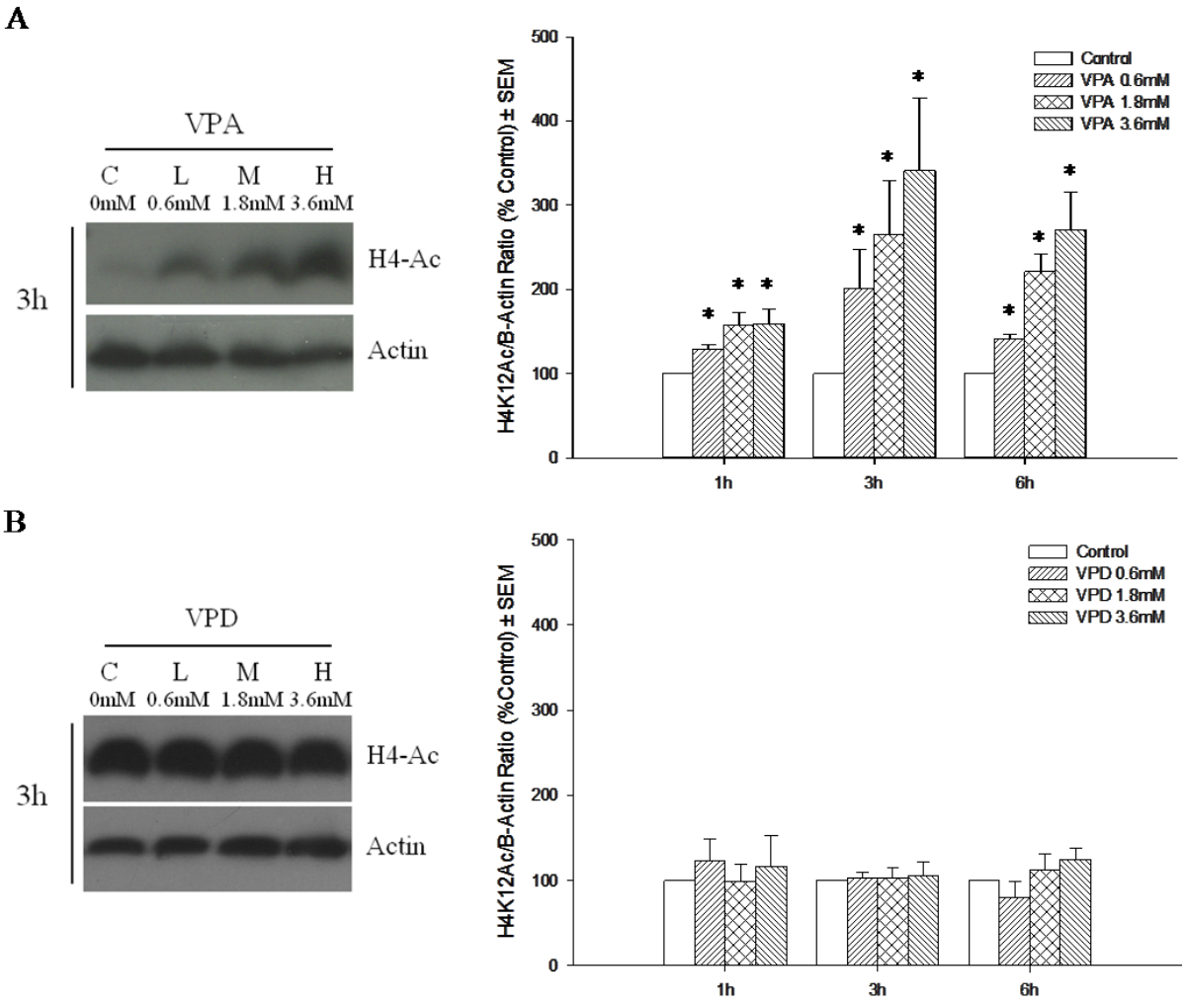
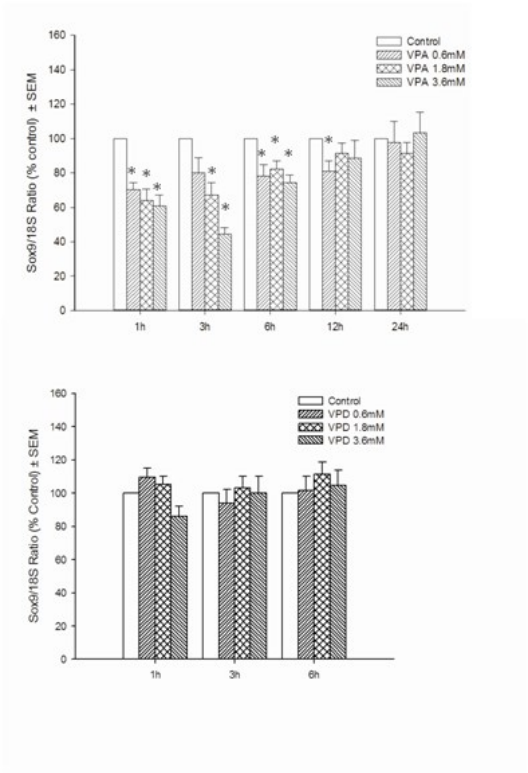


Figure 2.3 Sox9 gene expression following VPA (A) or VPD (B) treatment.

Limbs were cultured with VPA for 1, 3, 6, 12 or 24h and with VPD for 1, 3 or 6h. Sox9 mRNA was quantified by qRT-PCR and normalized to 18S rRNA. Results were subsequently normalized to the control. * = $p<0.05$



Effects of VPA and VPD on markers of chondrogenesis

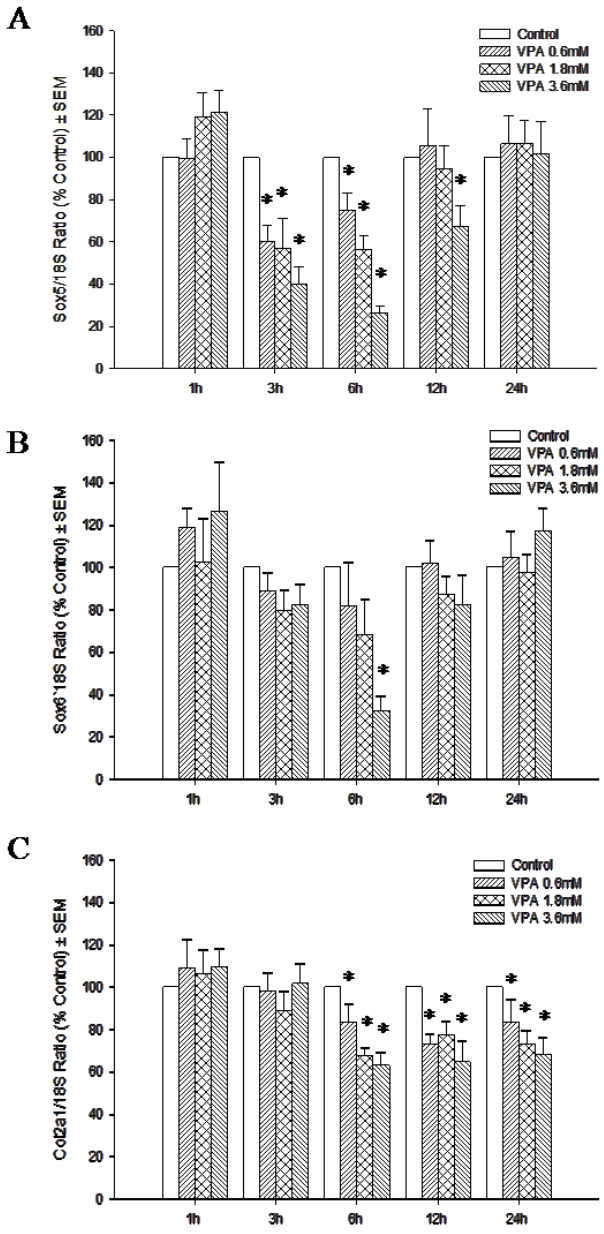
VPA exposure resulted in the downregulation of *Sox9* mRNA expression by 1h in all VPA-exposed limbs (Figure 2.3A). This inhibition peaked at 3h; transcripts returned to control levels in the limbs exposed to higher concentrations by 12h although histone-4 acetylation remained upregulated up to 24h (Figure S2.1), suggesting that a feedback mechanism helps to maintain homeostasis. VPD-treated sample did not show any changes in *Sox9* expression (Figure 2.3B).

SOX9 downstream targets, *Sox5* and *Sox6*, were downregulated after 3 and 6h, respectively (Figure 2.4A and B). *Col2a1* expression was significantly diminished in all VPA treated limbs by 6h; this inhibition persisted up to 24h following exposure (Figure 2.4C). This sequential inhibition of SOX9 downstream targets is consistent with the disruption of the Sox9-Sox5-Sox6 complex regulating Collagen 2 expression previously reported, suggesting that Sox signaling is inhibited by VPA treatment. Interestingly, although its three downstream targets are dowregulated at 6h, SOX9 protein expression was unchanged at this time point (Figure S2.2). SOX9 also regulates *Vegf* gene expression in the limb, as the limb mesenchyme-specific *Sox9* knock-out mice showed a lack of Vegf expression and limb vasculature defects (Eshkar-Oren et al., 2009). However, *Vegf* expression remained constant in our model (Figure S2.3), suggesting that VPA affects markers of chondrogenesis, but not vascularization at this time during limb development.

In order to determine if the effects on *Sox9* mRNA levels were due to an increase in the degradation of the transcripts or a decrease in transcription, we used embryonic

Figure 2.4 Effects of VPA on Sox9 signaling and the expression of its downstream target genes.

Sox5 (A), Sox6 (B) and Col2a1 (C) transcripts were quantified by qRT-PCR at 1, 3, 6, 12 or 24h following exposure to VPA. Transcripts were normalized to the 18S rRNA transcript and results were normalized to the control. * = $p<0.05$



forelimbs to obtain a primary cell culture that was subsequently co-treated with VPA and actinomycin D, a transcriptional inhibitor (Figure 2.5). Steady state concentrations of *Sox9* mRNA were significantly decreased following VPA treatment in primary cultures. As expected, a decrease in transcripts was observed over time in samples treated with actinomycin D alone. No additional effect was observed when cultures were co-treated with VPA and actinomycin D; therefore, the effects of VPA on *Sox9* mRNA expression are transcription-dependent.

To further examine the functional implications of the rapid effects on the expression of marker genes, limbs were exposed to VPA for 24h and cartilage condensation was assessed using Alcian blue staining. Control limbs showed deep staining in the digital space of 3-4 digits as well as in the ulna and radius, indicating the initiation of chondrogenic condensations (Figure 2.6). Several limbs exhibited condensations of the prospective phalanges. VPA-exposed limb exhibited a drastic decrease or an absence of staining in multiple digits, suggesting a delay in this process as early as 24h following exposure.

Effects of VPA and VPD on markers of osteogenesis

To determine the effects of VPA and VPD on the differentiation of osteocytes, the mRNA transcripts of *Runx2* and its downstream gene, Collagen10a1 (*Col10a1*), were analyzed by qRT-PCR. VPA treatment resulted in a downregulation of the expression of both genes at 3h and 24 h following exposure in a concentration dependent manner (Figure 2.7A, Figure 2.8). VPD did not significantly alter *Runx2* expression (Figure 2.7B), although a trend was observed which correlated with the subtle decrease in limb score in the highest VPD concentration group. In order to determine if these changes in

Figure 2.5 Effects of actinomycin D on VPA-induced downregulation in primary limb bud cell cultures.

Cells were exposed to vehicle or 3.6 mM VPA with or without actinomycin D for 1 or 3h. Total RNA was extracted and qRT-PCR for *Sox9* and 18S rRNA was done. * = $p<0.05$ compared to control. # = $p<0.05$ compared to 1h homologous groups.

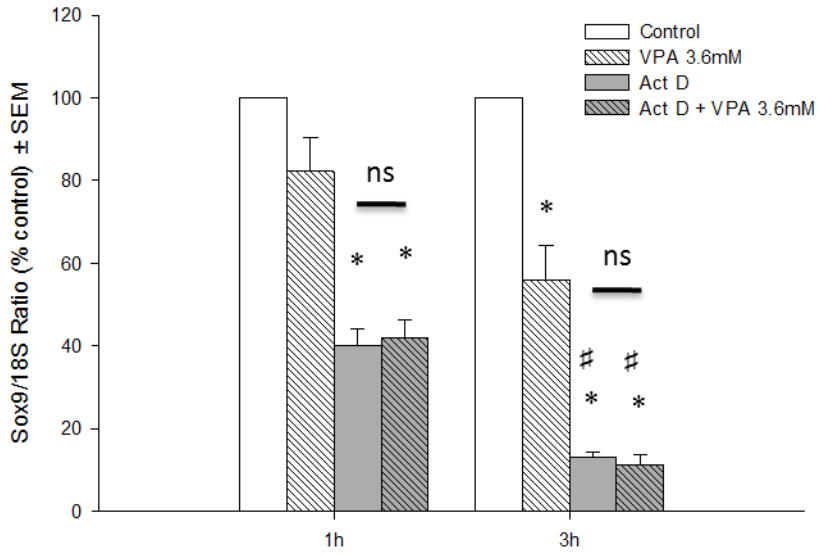


Figure 2.6 Effects of VPA exposure on early chondrocyte mesenchymal condensations.

Limbs were cultured for 24h in the presence of increasing concentrations of VPA and the cartilage proteoglycans were stained with alcian blue. Limbs were then mounted on slides and observed with light microscopy.

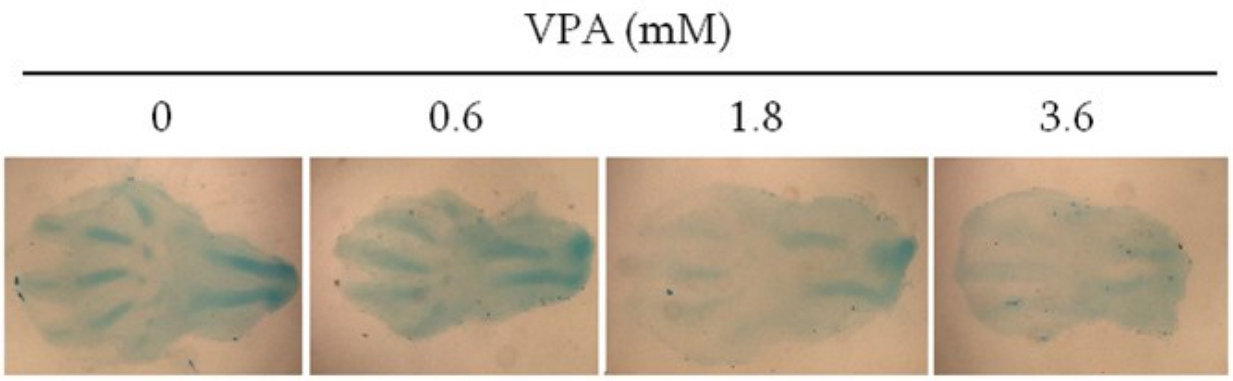


Figure 2.7 Effects of VPA and VPD on *Runx2* gene expression.

Total RNA was extracted from VPA-treated (A) and VPD-treated (B) limbs. *Runx2* transcripts were quantified and normalized to 18s rRNA. Results were further normalized to the control. * = $p<0.05$

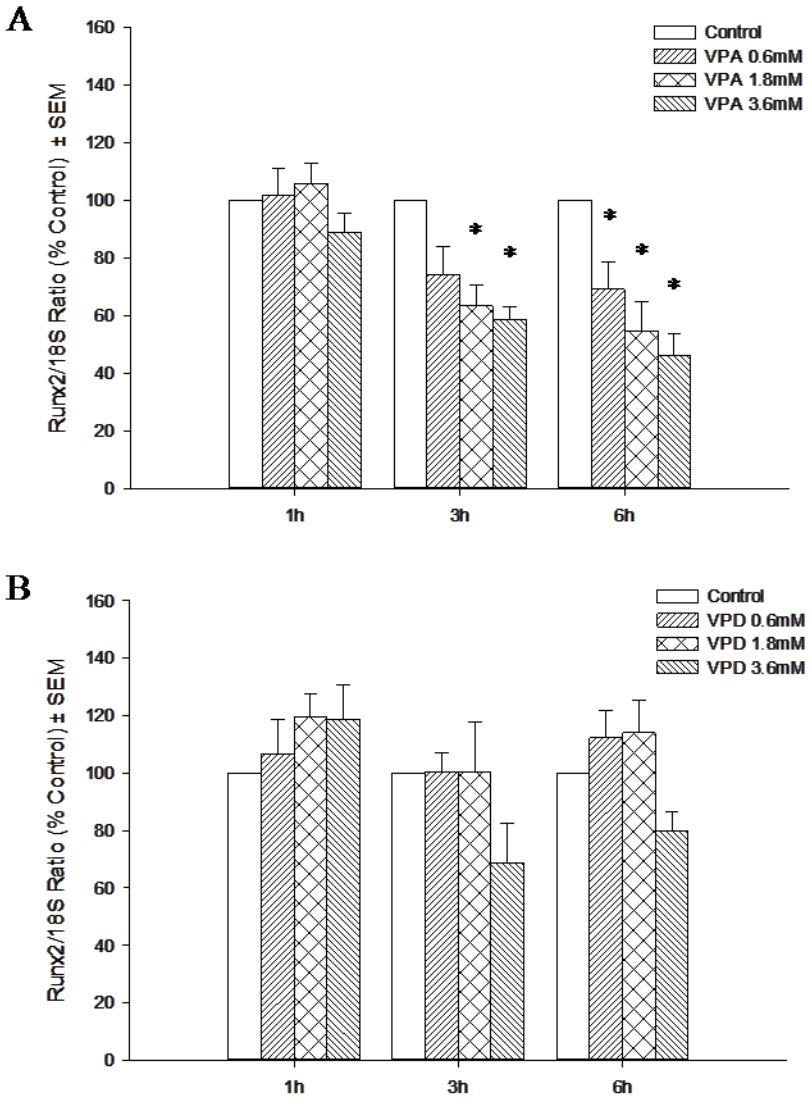
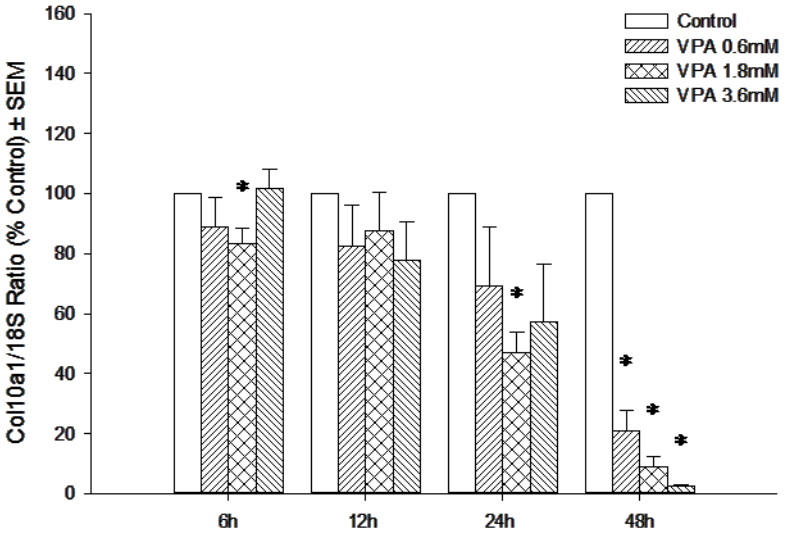


Figure 2.8 Effects of VPA on Runx2 downstream signaling.

At 6, 12, 24 and 48h, limb cultures were ended and total RNA was isolated. *Col10a1* mRNA was quantified and 18S rRNA was used for normalization. Results are expressed as % control. * = $p<0.05$



gene expression had an impact on bone mineralization, limbs were cultured for 6 days, as for the morphological analysis, and stained with alizarin red. However, no staining was observed, suggesting that this process is not detected in our in vitro system (data not shown)

2.4 Discussion

Although VPA is both a commonly used anticonvulsant and an established human teratogen, the mechanisms underlying these actions are complex and have not been resolved. This study provides the first evidence that the *Sox9* and *Runx2* signaling pathways are targeted in VPA-induced limb developmental defects. As *Sox9* is expressed in a number of tissues, VPA-induced downregulation of *Sox9* may affect the formation of many organs during embryogenesis. In humans, a heterozygous deletion of the *Sox9* gene results in a condition known as campomelic dysplasia that is often lethal due to thoracic cage defects and respiratory distress; survivors often exhibit sex reversal and severe skeletal and craniofacial malformations, such as cleft palate, scoliosis and micrognatia. Some cases had spina bifida, global developmental delay and learning impairment, or developed seizures later in life, suggesting that neural development is impaired by *Sox9* deletion (Mansour, Offiah et al. 2002). Hence, the deregulation of this pathway may be involved in multiple defects.

The mechanism by which VPA exposure leads to the transcriptional downregulation of *Sox9* expression is not known. The rapidity of the response suggests that it is independent of *de novo* protein synthesis. Our results showed that this effect is dependent on transcription and point toward a direct modulation of transcriptional

regulation through post-translational modifications. The VPA-induced induction of histone 4 hyperacetylation and inhibition of *Sox9* expression are directly correlated, both with respect to timing and concentration-dependence. Furthermore, VPD, a close analog of VPA, failed to affect either histone acetylation or *Sox9* expression. HDAC inhibition may affect several pathways that are important in chondrogenesis. Two other HDAC inhibitors, trichostatin A and PDX101, were reported to repress Collagen 2 expression in rabbit articular chondrocytes in a Wnt5a-dependent manner (Huh, Ryu et al. 2007). These studies are consistent with our results as *Col2a1* expression was repressed in VPA-exposed limbs.

In addition to Wnt signaling, several pathways regulating *Sox9* expression have been identified; these include the bone-morphogenetic protein (BMP), nuclear factor kB (NF-κB) and p38 MAPK pathways (Murakami, Kan et al. 2000, Pan, Yu et al. 2008). Downregulation of *Bmp2* and upregulation of the inhibitory *Smad7* were observed following trichostatin A-induced HDAC inhibition, ultimately leading to a downregulation of BMP signaling (Shaked, Weissmuller et al. 2008). Moreover, although histone tails and chromatin compaction have been considered the primary targets of HDACs, several non-histone protein targets have now been identified. Among these are many *Sox9* transcriptional regulators that are modulated by acetylation. For example, SMAD7 was shown to directly interact with HDAC1; trichostatin A treatment caused SMAD7 hyperacetylation and stabilization. Both the p65 and p52 subunits of the NFκB complex can be acetylated and this acetylation modifies NFκB transcriptional activity (Hu and Colburn 2005, Simonsson, Heldin et al. 2005, Kim, Jang et al. 2012).

VPA treatment repressed the expression of *Runx2*, downstream of the *Sox9* pathway in chondrocyte differentiation and limb development. Although VPD had no effect on *Sox9* expression, limbs exposed to the highest concentration of VPD showed a non-significant decrease in *Runx2* expression at both 3 and 6h. It is notable that there was a small decrease in limb score in this treatment group, in the absence of any detectable increase in histone acetylation. It is possible that any effect of VPD on *Runx2* expression does not involve HDAC inhibition. While we have observed a repression in *Runx2* expression in our limb buds in culture, other groups have reported that VPA and other HDAC inhibitors induce *Runx2* in mesenchymal stem cells and calvarial-derived primary osteoblasts cells, thus promoting osteogenic differentiation (Cho, Park et al. 2005, Schroeder, Jensen et al. 2005). This discrepancy suggests that *Runx2* regulation differs in whole limb buds and in cell culture and may be tissue and developmental stage-specific. Altogether, this study provides valuable insight into the mechanism of action of VPA during limb development. Identifying molecular pathways affected by this teratogen and other birth defect-inducing drugs is pivotal for the development of assays to test the potential of drugs as teratogens before they are prescribed to women who are pregnant or of child-bearing age.

2.5 Supplementary Data description

Supplementary figures are provided for the effects of VPA on histone H4 acetylation after 12 or 24 h in culture, on SOX9 protein concentrations at 6h, and on *Vegf* gene expression.

2.6 Funding Information

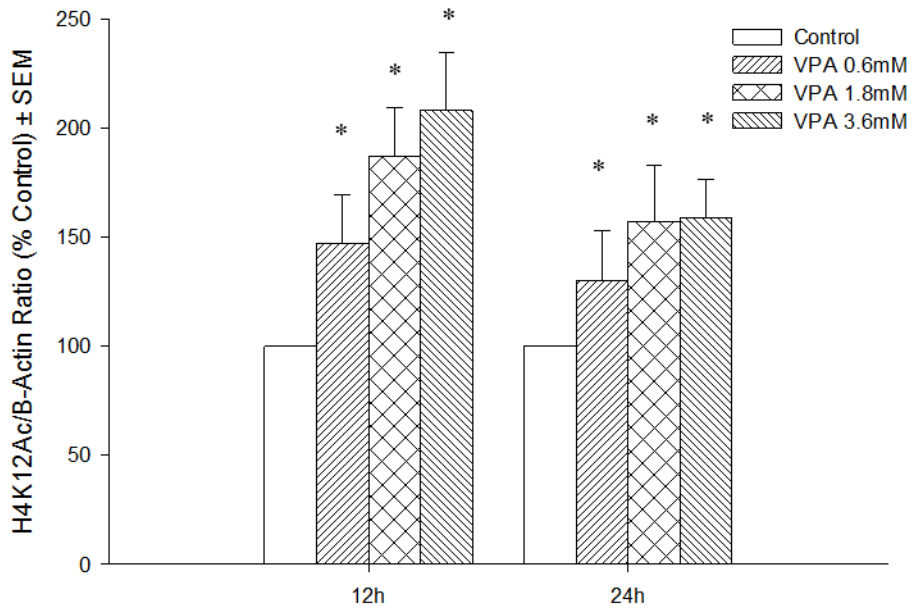
This work was supported by CIHR grant MOP-86511. FH Paradis received funding from CIHR and FRSQ. BF Hales is the recipient of a James McGill Professorship

2.7 Acknowledgements

We should like to thank Katwijk Chemie for providing Valpromide.

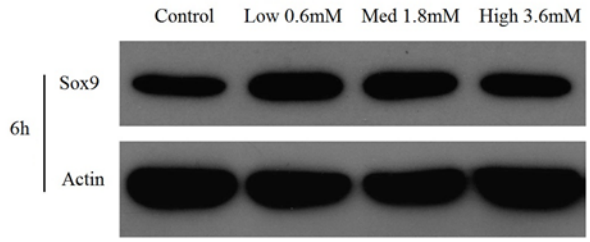
Supplementary Figure S 2.1 Histone-4 hyperacetylation is maintained at 12 and 24h.

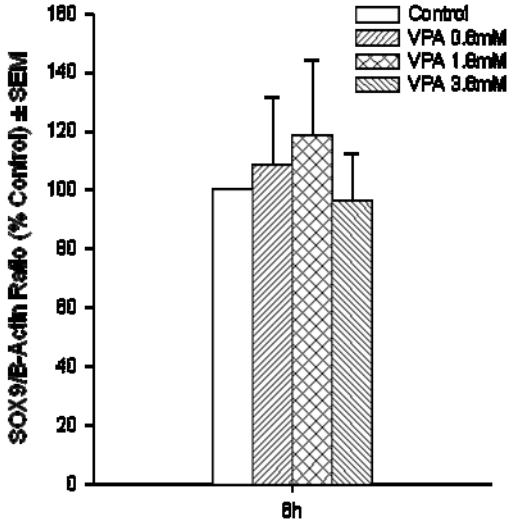
H4K12 acetylation was used as a marker of HDAC inhibition and normalized to β-Actin. Western blot of whole cell protein extracts from VPA-treated limbs at 12 and 24h following exposure. Data were quantified by densitometry. * = $p<0.05$



Supplementary Figure S 2.2 Effects of VPA on SOX9 protein.

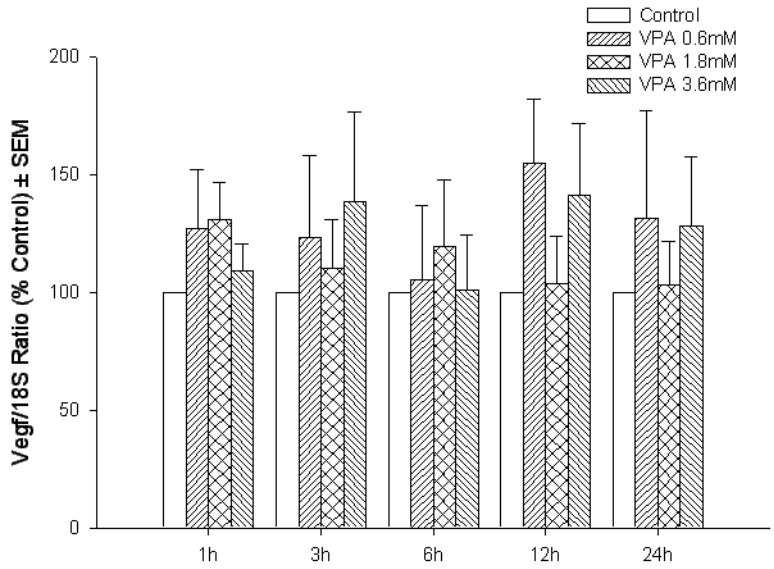
Whole-limb lysates were obtained from VPA-treated limbs at 6h. Proteins were separated by gel electrophoresis, transferred to PVDF membranes and probed using SOX9 antibody.





Supplementary Figure S 2.3 *Vegf* gene expression after VPA exposure.

Limb cultures were arrested at 1, 3, 6, 12 or 24h and analyzed by qRT-PCR. *Vegf* transcripts were sequentially normalized to 18S rRNA and to control.



Connecting Text

The data presented in Chapter 2 show the effects of VPA on limb morphology and signaling pathways involved in chondrogenesis and osteogenesis. As apoptosis both follows and precedes chondrocyte hypertrophy and osteoblast differentiation, respectively, the effects of VPA on cellular death are investigated in Chapter 3. Specifically, the p53 signaling pathway was chosen among the various apoptotic signaling pathway because of its importance as a pro-apoptotic factor and high expression in the mid-organogenesis limb bud.

Chapter 3 Valproic Acid Induces the Hyperacetylation and Activation of P53 and the Intrinsic Apoptotic Pathway in Mid-Organogenesis Murine Limbs

France-Hélène Paradis and Barbara F. Hales

Manuscript in preparation

3.1 Abstract

Exposure to valproic acid (VPA), an anticonvulsant and histone deacetylase inhibitor (HDACi), increases the risk of congenital malformations. Although the mechanisms leading to the teratogenicity of VPA remain unsolved, several HDACi cause programmed cell death in cancer cell lines. Moreover, p53, the master regulator of apoptosis, is an established HDAC target. The purpose of this study was to investigate the effects of VPA on p53 signaling and markers of apoptosis during mid-organogenesis limb development. Timed-pregnant CD1 mice (GD 12) were euthanized; embryonic forelimbs were excised and cultured in vitro for 3, 6, 12 and 24 h in the presence or absence of VPA or valpromide (VPD), a non-HDACi analog of VPA. Quantitative RT-PCR and western blots were used to assess the expression of candidate genes and proteins involved in p53 signaling and apoptosis. P53 hyperacetylation and transcriptional activation were observed only in VPA-treated limbs. VPA exposure also activated markers of apoptosis, including the levels of cleaved caspase 9 and 3 and cleaved-poly (ADP-ribose) polymerase (PARP). VPD treatment caused a small, although significant, increase in cleaved caspase 3. The DNA damage marker, γ-H2AX was also increased following VPA exposure. These data indicate that p53-activation via acetylation triggers an increase in apoptosis that may contribute to the teratogenicity of VPA and other HDACi.

Keywords:

Valproic acid, Valpromide, HDAC inhibitor, teratogen, limb development, p53, apoptosis

3.2 Introduction

Valproic acid (VPA), first discovered as an anticonvulsant in 1963, is now commonly used world-wide to treat epilepsy (Meunier, Carraz et al. 1963), bipolar disorders, and for prophylaxis against migraines (Evers 2008, Bowden 2009). It was identified recently as a potential anticancer agent and has been shown to increase the efficacy of 5-azacytidine, the DNA-methyltransferase inhibitor, in combination therapy for myelodysplasic syndromes (Voso, Santini et al. 2009). However, when taken during pregnancy, VPA readily crosses the placenta and accumulates in the developing embryo where it disrupts organogenesis, leading to a variety of birth defects, including neural tube and skeletal anomalies (Nau and Scott 1986).

The anticonvulsant ability of VPA is attributed to its action on different enzymes of the GABA degradation pathways as well as on sodium channels in the synapse (Van den Berg, Kok et al. 1993, Johannessen and Johannessen 2003). However, the mechanism of action underlying its other pharmacological activities, including its teratogenesis, remains unresolved.

Recent studies have suggested that VPA-induced teratogenesis is linked to its induction of DNA damage and its activity as a histone deacetylase (HDAC) inhibitor (Menegola, Di Renzo et al. 2006, Tung and Winn 2011, Paradis and Hales 2013). More than 14 HDAC inhibitors are currently in clinical trials as anti-neoplastic agents and three, vorinistat/SAHA, romidepsin and belinostat have been approved by the Food and Drug Administration for the treatment of T-cell lymphoma (Rangwala, Zhang et al. 2012, Bodiford, Bodge et al. 2014). A central mechanism of action of these drugs appears to be the induction of apoptosis (Shankar and Srivastava 2008). As an increase in

apoptosis is involved in the teratogenicity of many drugs, including ethanol, retinoids, and anticancer drugs such as cyclophosphamide, we hypothesize that VPA-mediated HDAC inhibition results in cellular death in the developing embryo, leading to birth defects (Huang and Hales 2002, Ali-Khan and Hales 2003).

HDACs interact with both histone and non-histone protein targets, including a number of transcription factors, such as NF-kB, GATA(1-4), HIF1A and p53 (Glozak, Sengupta et al. 2005). P53 is a well-studied pro-apoptotic tumor suppressor transcription factor. Its regulation and activation are extremely complex and involve input from a variety of pathways (Lavin and Gueven 2006). Under normal physiological conditions and in the absence of stress, p53 is constantly degraded and maintained at very low levels by its major repressor, the E3 ubiquitin ligase Mdm2. Under conditions that induce stress and DNA damage, p53 is modified via post-translational changes, including acetylation, leading to its release from Mdm2 and its stabilization (Lavin and Gueven 2006). The p53 C-terminal regulatory domain contains multiple lysine residues that are acetylated by p300 and PCAF, leading to increased DNA binding activity (Liu, Scolnick et al. 1999, Luo, Su et al. 2000, Lavin and Gueven 2006). Activated p53 translocates to the nucleus where it regulates the expression of several pro- and anti-apoptotic genes, such as *bax*, *bcl2*, *puma* and *survivin (birc5).* Additionally, it plays a role in DNA damage/repair pathways and in cell cycle regulation and directly regulates the expression of *p21/cip1 (Cdk-interacting protein 1)*.

P53 plays a role during embryonic development and in teratogen-mediated birth defects in addition to its activity as a tumor suppressor gene. In mid-organogenesis stage embryos, *p53* is highly expressed in the limb buds yet little is known about its action in

limb organogenesis (EMAGE gene expression database (http://www.emouseatlas.org/emage/) (Richardson, Venkataraman et al. 2014). Although p53 knockout mice develop normally and live up to four months of age, mice expressing a truncated p53 lacking the C-terminal end exhibit a plethora of defects, including kinked tails and significantly smaller tibias. Mice lacking the *mdm2* gene, in which p53 is constantly activated, die during embryogenesis at gestational day 6.5; no mdm2 limb conditional knock-outs exist to our knowledge (Montes de Oca Luna, Wagner et al. 1995, Jonkers, Meuwissen et al. 2001, Hamard, Barthelery et al. 2013). In addition, p53 is involved in PDGFRa signaling-mediated increased survival and proliferation during skeletal development (Fantauzzo and Soriano 2014). In this study, we tested the hypothesis that VPA treatment affects both p53 signaling and cellular apoptosis in the developing limb. Using an in vitro limb bud culture system, we report here that VPA exposure induces p53 hyperacetylation and activation and triggers the intrinsic apoptotic cascade. In contrast, valpromide (VPD), a VPA analog that does not inhibit HDACs induces only mild effects on limb development (Paradis and Hales 2013), does not affect p53 activation but causes a slight increase in apoptosis. Altogether, these data suggest that changes in p53 activation have an impact on multiple signaling pathways and that VPA-induced apoptosis is multifactorial.

3.3 Material and Methods

Limb bud cultures and drug treatments

Timed-pregnant CD1 mice (20-25 g), purchased from Charles River Canada Inc. (St-Constant, QC, Canada), were euthanized by cervical dislocation on gestation day 12 and their embryos were explanted. All animal studies complied with the guidelines established by the Canadian Council on Animal Care under protocol 1825. The embryonic forelimbs were cultured, as previously described (Huang and Hales 2002). Briefly, limbs were excised in Hank's balanced salt solution, pooled and cultured in vitro in 6 ml culture medium consisting of 75% BGJb medium (GIBCO BRL Products, Burlington, ON, Canada), 25% salt solution supplemented with ascorbic acid (160 µg/ml), and gentamycin (1 µl/ml, GIBCO BRL Products). Each culture was gassed with 50% O_2, 5% CO_2 and 45% N_2. Different concentrations of sodium valproate (VPA, Sigma, St-Louis, MO, #P4543) or valpromide (a gift from Katwijk Chemie, Netherlands) dissolved in distilled water and DMSO, respectively, were added to designated cultures.

RNA preparation and real-time qRT-PCR

Total RNA from homogenized limbs (3-4 limbs per group) was extracted using an RNeasy Microkit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's specifications. RNA concentrations were determined by spectrophotometric analysis using a Nanodrop1000 spectrophotometer (Thermo Scientific, Nepean, ON, Canada). A 260:280 absorbance ratio of 2.0 ± 0.2 was used as an indicator of the RNA purity. The samples were diluted to a working concentration of 1 ng/µl and transcripts were quantified using Quantitect One-Step SYBR Green RT-PCR (Qiagen) and the Applied Biosystems Step One Plus Real-time PCR system (Burlington, ON, Canada). Primer sets were purchased from Qiagen for *Bcl2* (#QT02392292), *Cdkn1a*/*p21* (cyclin-

dependent kinase inhibitor 1) (#QT00137053), *Birc5* (Survivin) (#QT00113379), and *18S rRNA* (#QT01036875). PCR was done under the following conditions: 20 min at 50°C, 40 cycles of 95°C for 15 min, 94°C for 15s, 55°C for 30s, and 72°C for 20s. Each reaction was done in triplicate, averaged and normalized to the amount of 18S rRNA transcripts. Each experiment was replicated 6-10 times per group (n=6-10).

Western blotting

Cultures were ended at specified times. Limbs (7-8 limbs per group) were homogenized by sonication in lysis buffer containing protease inhibitors: 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5, 40 µg/ml bestatin, 0.2 M phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 6 µg/ml aprotinin. Total protein was extracted and quantified using spectrophotometric Bio-Rad protein assays (Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins (15-30 µg per sample) were separated by SDS-PAGE acrylamide gel electrophoresis and transferred on to polyvinylidene difluoride membranes (BioSciences Inc., Baie d'Urfé, QC, Canada). Precision standards (Bio-Rad Laboratories) were used as molecular weight markers. Membranes were blocked in 5% non-fat milk in TBS-T (137 mM NaC1, 20 mM Tris pH 7.4, 0.05% Tween 20) for 1 h at room temperature, probed overnight at 4°C with primary antibodies, washed and incubated with the secondary antibody for 2 h at room temperature. Immunoblotting was done using antibodies against P53-K379-Ac (Cell Signaling, Danvers, MA, USA, 1:500), p53 (Cell Signaling, 1:1000), cleaved-caspase 9 (Cell Signaling, 1:1000), cleaved-caspase 3 (Cell Signaling, 1:500), cleaved-poly (ADP-ribose) polymerase (PARP) (Cell Signaling, 1:1000), γ-H2A.X (Cell Signaling, 1:1000)

and beta-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:10000). The secondary antibodies, conjugated to horseradish peroxidase (HRP), were donkey anti-rabbit antibodies (GE Healthcare Limited, Baie d'Urfé, QC, Canada, 1:5000), anti-mouse antibodies (GE Healthcare Limited, 1:5000) and anti-goat antibodies (Santa Cruz Biotechnology, Inc, 1:10000). Western blots were visualized with the Enhanced Chemiluminescence Plus Kit (GE Healthcare Limited) and quantified by densitometry using a Chemi-Imager 400 imaging system (Alpha Innotech, San Leandro, CA). Each experiment was replicated 5 times per group.

Statistical Analyses

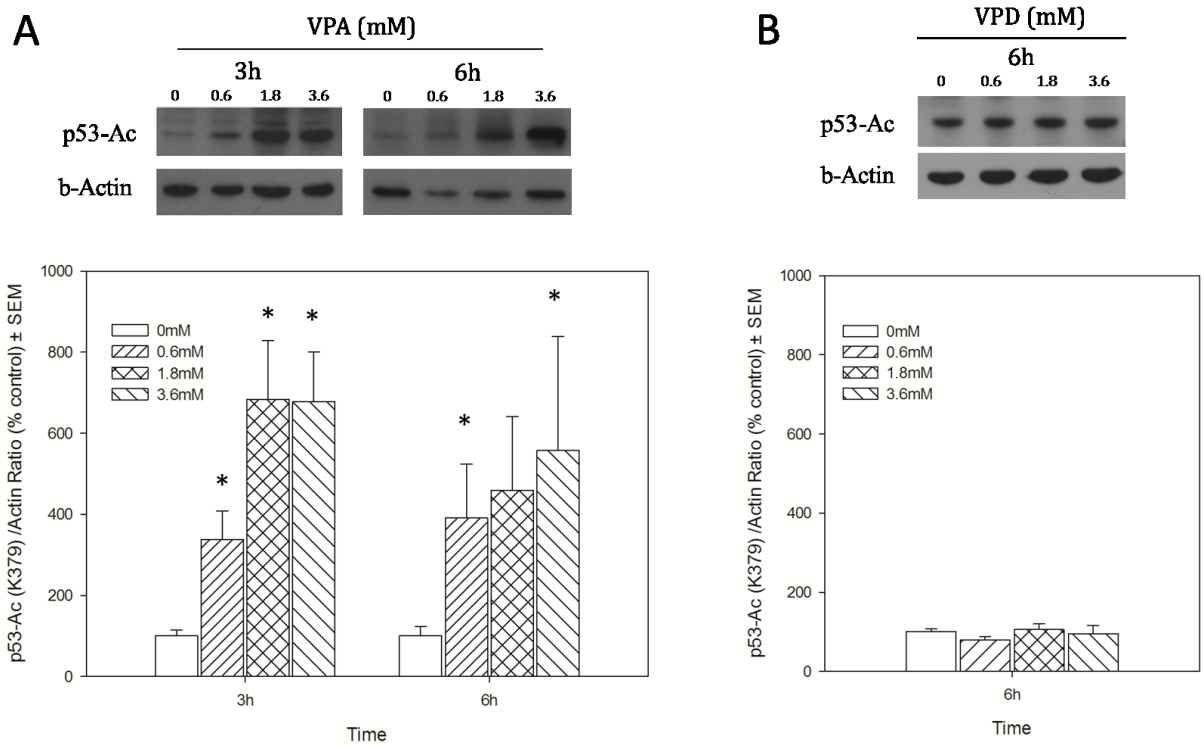
All data sets were analyzed statistically using SigmaPlot 11.0 (Systat Software, Point Richmond, CA). The Ranked Mann-Whitney U test and Bonferonni's multiple-comparison correction were used to compare all concentration groups to the controls within a given time point. The minimum level of significance was $P < 0.05$.

3.4 Results

To assess the effects of VPA on p53 acetylation in the developing limb, proteins were extracted from limbs exposed to VPA for different periods of time and were analysed by western blot. VPA induced a significant increase in p53 acetylation following exposure for 3 or 6 h (Fig. 3.1 A), whereas treatment with VPD had no effect (Fig. 3.1 B). p53 levels were unchanged (Figure S3.1). These results suggest the HDACs targeting p53 are present in the limb at this stage of development and are inhibited by VPA but not VPD.

Figure 3.1 p53 is hyperacetylated following VPA but not VPD exposure.

A. Limbs were treated with VPA (0, 0.6, 1.8 or 3.6 mM) for 3 or 6 h and whole cell lysates were subsequently extracted. Proteins were separated by electrophoresis, transferred onto a PVDF membrane and probed with a specific antibody to p53-Ac (K382) (Upper panel). Actin was used as a loading control. Bands were quantified by densitometry (Lower panel). B. The same experiment was conducted with limbs exposed to VPD (0, 0.6, 1.8 or 3.6 mM) * = $p<0.05$, n=5-6 (7-8 limbs per group per n).



To query the effects of VPA-induced p53 hyperacetylation on p53 activation in treated limbs, the levels of different transcriptional targets of p53 were analysed by qRT-PCR. Significant differences were observed in all treatment groups. VPA treatment significantly downregulated the expression of *Bcl2* and *Surv/Birc5*, two genes negatively regulated by p53, at 3 and 6h (Fig. 3.2 A, C) whereas *p21/cip1,* a positively regulated p53 target, was upregulated at 3h following exposure (Fig. 3.2 B). Together, these results suggest that the transcriptional activity of p53 is increased by VPA treatment; this may lead to an increase in its pro-apoptotic activity.

We further investigated the activation of the intrinsic apoptotic signaling cascade (Fig.3.3). VPA treatment increased caspase 9 cleavage, starting at 6 h and reaching significance at 12 h. The cleavage of its downstream target, caspase 3, was also enhanced at 12h in the two highest VPA treatment groups; a small increase in cleaved-caspase 3 was also observed in the highest concentration VPD treatment group. Finally, VPA exposure increased the cleavage of poly-ADP-ribose polymerase (PARP), a recognized marker of cellular apoptosis and target of activated-caspase 3, at 12 h. These results suggest that VPA triggers activation of the intrinsic apoptotic pathway, leading to cellular apoptosis.

As DNA damage activates p53, we investigated the effects of VPA and VPD on the DNA double-strand breaks marker γH2A.X. γH2A.X expression was not affected by VPA treatment at 3h but by 12h a drastic concentration-dependent increase in γ-H2A.X expression was observed; VPD exposure did not significantly affect γ-H2A.X concentrations at 12h (Fig. 3.4). The time course of the VPA-induced increase in DNA

Figure 3.2 VPA affects the expression of p53 downstream targets

RNA was purified from 3-4 limbs treated with VPA; quantitative RT-PCR was used to quantify transcript levels of different genes (A. Survivin, B. p21, C. Bcl2) that were then normalized to the *18S* rRNA levels. * = $p<0.05$, n=6-10 (3-4 limbs per group per n).

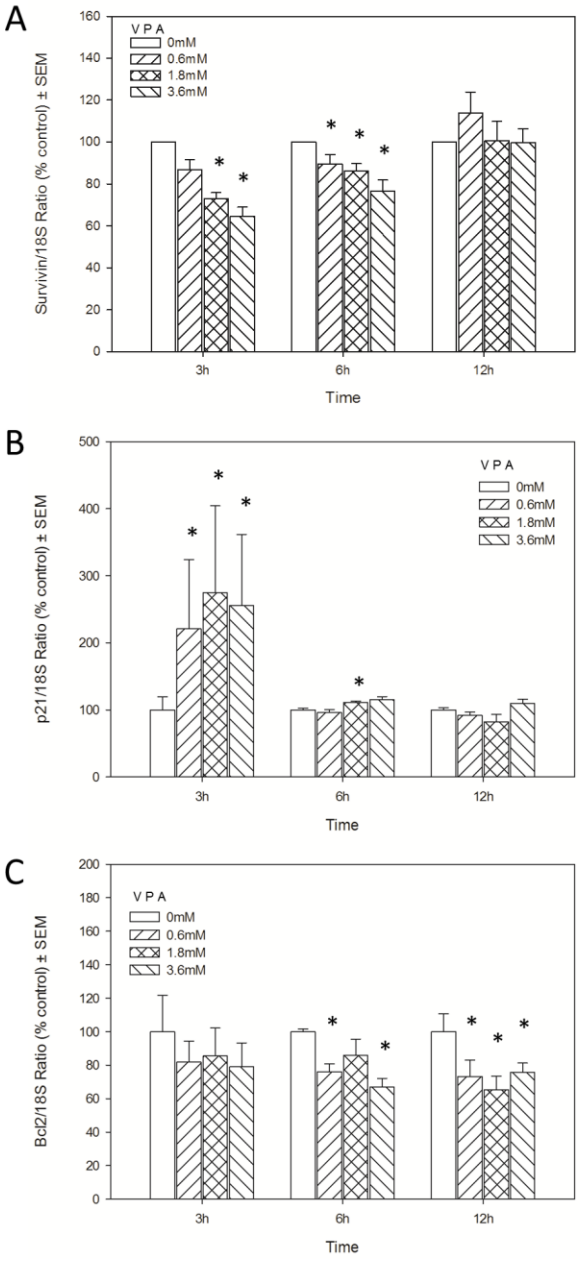


Figure 3.3 The intrinsic apoptotic caspase cascade is triggered following VPA exposure.

A, B and C. Whole cell lysates were extracted from limbs cultured for the indicated times in the presence of VPA (0, 0.6, 1.8 or 3.6mM). Proteins were separated by electrophoresis and proteins were detected by immunoblotting (Upper panel)(A. Cleaved-caspase 9, B. Cleave. Actin was used as a loading control. Bands were quantified by densitometry (Lower panel). D. Cleaved-caspase 3 protein level were measured in limbs exposed to VPD (0, 0.6, 1.8 or 3.6mM)* = $p<0.05$, n=5 (7-8 limbs per group per n).

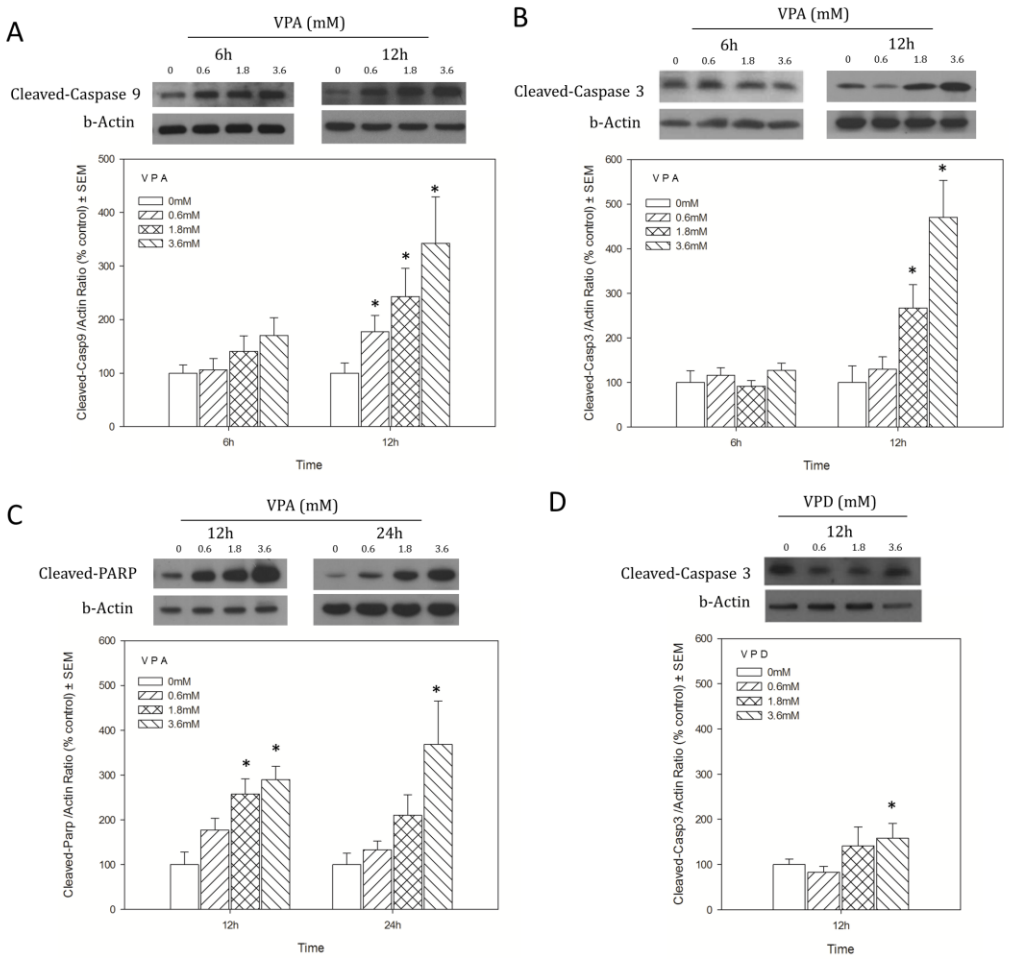
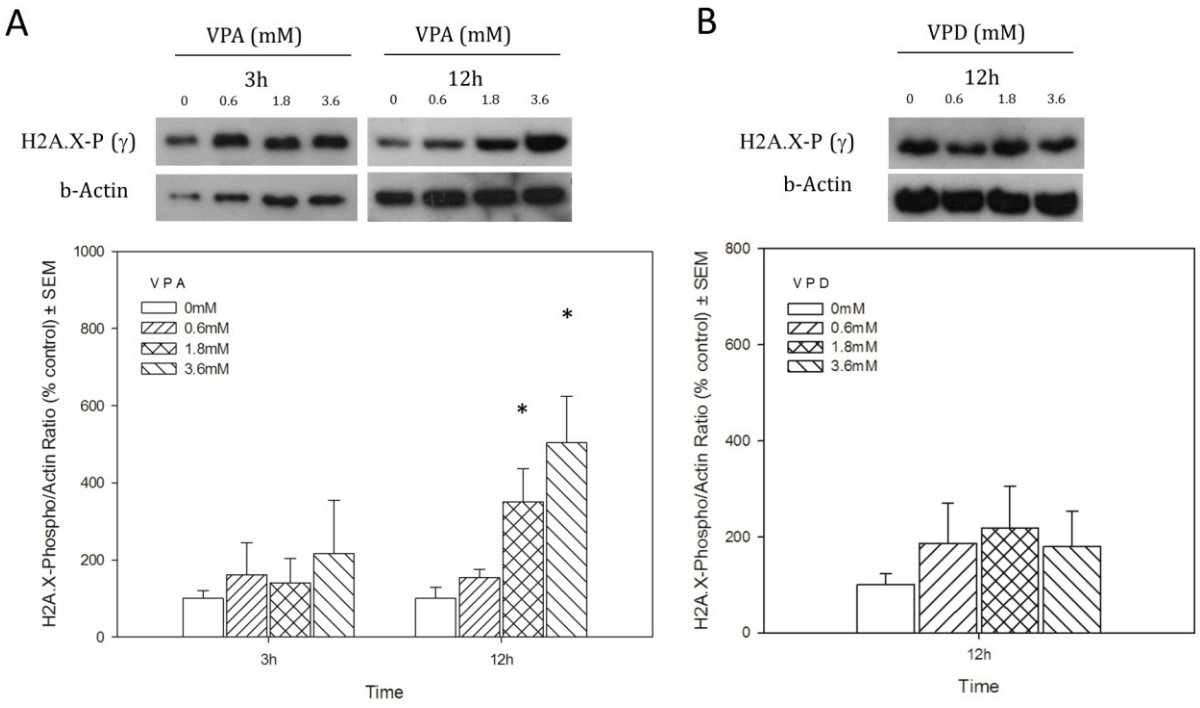


Figure 3.4 VPA treatment increases DNA damage.

Proteins from whole limb lysates were extracted, separated on an SDS-page gel and transferred onto a PVDF membrane. A specific antibody against γH2A.X and actin were used to detect the protein levels at 12 h following VPA (A) or VPD (B) exposure (Upper panel). Bands were quantified by densitometry (Lower panel). * = $p<0.05$, n=5 (7-8 limbs per group per n).



damage suggests that it does not contribute to the VPA-induced increase in p53 activation at 3h.

3.5 Discussion

Here we show that VPA exposure leads a concentration dependent p53 hyperacetylation and sequential activation, triggering the intrinsic apoptotic pathway in the mid-organogenesis stage murine limb. Tight regulation of p53 is pivotal for the proper development of multiple organs (Montes de Oca Luna, Wagner et al. 1995, Liu, Terzian et al. 2007). The importance of p53 regulation during embryogenesis is readily demonstrated in transgenic mice in which p53 hyperactivation, due to elimination of the p53 domain that interacts with mdm2 or of mdm2 itself, leads to mortality early during embryogenesis.

In addition to their role in the intrinsic pathway, p53 and caspase 3 are involved in regulation of the extrinsic pathway, characterized by activation of the Fas receptor and caspase 8. The ability of VPA to activate the extrinsic apoptotic pathway may be dependent on the cells studied. VPA triggered the extrinsic apoptotic pathway via the TRAIL-ligand, leading to the activation of caspase 8, in chronic lymphocytic leukemia cells (Lagneaux, Gillet et al. 2007). However, in neuroblastoma cell lines, VPA triggered apoptosis in a completely caspase 8-independent manner (Cipro, Hrebackova et al. 2012). Previous studies from our lab with other teratogens, retinol and activated cyclophosphamide, suggest that in limbs during organogenesis, caspase 8 cleavage

and the activation of the extrinsic pathway is not a major target of developmental toxicants (Huang and Hales 2002, Ali-Khan and Hales 2003)

Tung and Winn showed an increase in caspase 3 activation in the somites of gestational day 9 embryos treated with VPA in a whole embryo culture system that is consistent with the results of this study. In contrast with the increase in double-strand break marker phosphorylated H2A.X that we observed, in their model, they did not find an increase in oxidative DNA damage marker as assessed by 8-hydroxyguanosine (8-OHdG) (Tung and Winn 2011). Also, gestation day 8 embryos exposed to VPA in vivo exhibited an increase in apoptosis that did not coincide with an increase in caspase 3 activation (Di Renzo, Broccia et al. 2010). Altogether, these data suggest that VPA-mediated cellular apoptosis is influenced by multiple pathways modulated by p53 and might be triggered in a tissue and time-dependent manner in the developing embryo.

In a previous study from our laboratory we showed that the effects of VPA and VPD on limb morphology were highly correlated with their HDAC inhibitory activity; VPA caused a concentration-dependent increase in both limb malformations and histone hyperacetylation (Paradis and Hales 2013). The concentration-dependent effects of VPA on p53 signaling, the apoptosis cascade and γH2A.X, together with the absence of effects in VPD-treated limbs, further suggest an association between HDAC inhibition and p53 activation.

In a number of studies, micro-RNAs have been shown to be regulators of apoptosis, including members of the miRNA-34 family that are direct targets of p53 (He, He et al. 2007). Recently, Gueta et al. showed that limbs exposed to the teratogen

cyclophosphamide exhibited limb malformations that correlated with both increased expression of these miRNAs and the p53 genotype of the mice. These data suggest that p53 plays a central role in cyclophosphamide mediated effects (Gueta, Molotski et al. 2010). Moreover, MiR34a acts as a negative regulator of mesenchymal cell differentiation into chondroblasts (Kim, Song et al. 2011). As VPA inhibits chondrogenesis as well as induces apoptosis, miR34 is an interesting candidate as a central target of teratogens.

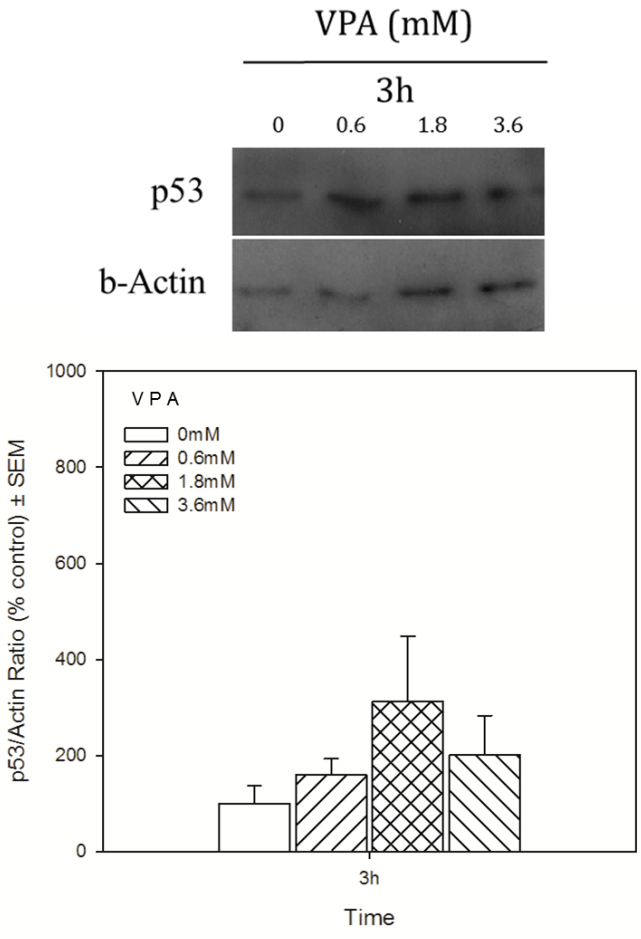
In summary, we have shown that VPA exposure triggers the hyperacetylation and sequential activation of p53 signaling and the mitochondrial intrinsic caspase cascade, leading to an increase cell death in mid-organogenesis limbs. This study suggests that p53 is a direct target of VPA, and possibly as a central target of HDACi, during embryogenesis and broadens our understanding of the molecular mechanisms leading to HDACi-induced teratogenesis.

3.6 Acknowledgements

We should like to thank Katwijk Chemie for providing Valpromide. This work was supported by CIHR grant MOP-86511. FH Paradis received funding from CIHR and FRSQ. BF Hales is the recipient of a James McGill Professorship.

Supplementary Figure S3.1 P53 protein expression remains unchanged following VPA exposure.

Proteins from whole limbs lysate were extracted, separated on an SDS-page gel and transferred onto a PVDF membrane. A specific antibody against p53 and actin were used to detect the protein levels at 3h following VPA exposure. n=5 (7-8 limbs per group per n).



Connecting Text

The data presented in Chapter 2 and 3 show the effects of VPA, an inhibitor of class I and II HDACs, on three critical processes involved in development: chondrogenesis, osteogenesis and apoptosis. In the next chapter, we explored the specific effects of inhibitors of the different HDAC classes on these processes to further investigate the upstream mechanisms leading to the effects; MS275 (class I inhibitor), MC1568 (class II inhibitor) and Sirtinol (class III inhibitor) were used.

Chapter 4 Class I and III histone deacetylase (HDAC) inhibitors are embryotoxic in mid-organogenesis murine limb buds in culture

France-Hélène Paradis and Barbara F. Hales

4.1 Abstract

Histone deacetylases (HDACs) play a major role in chromatin remodeling, gene regulation and cellular signaling. Several inhibitors of these enzymes have been shown to cause embryotoxicity and increase the risk of birth defects. The role of each class of HDAC during normal development is unclear and the mechanisms leading to their teratogenicity remain unsolved. The purpose of this study was to investigate the effects of class-specific HDAC inhibitors (HDACi) on limb formation and bone differentiation during organogenesis. The differentiation of proliferative chondrocytes, hypertrophic chondrocytes and osteoblasts was assessed using Col2a1, Col10a1 and Col1a1, respectively, as biomarkers. Timed-pregnant Col2a1-ECFP, Col10a1-mCherry and Col1a1-YFP CD1 reporter mice were euthanized on gestation day 12; embryonic forelimbs were excised and cultured in vitro for 1, 3, and 6 days in the presence or absence of MS275 (Class I HDACi), MC1568 (class III HDACi), sirtinol (Class II HDACi), or valproic acid (VPA), our positive control. Fluorescent microscopy was used to examine the expression of fluorescently-tagged markers. MS275 and VPA caused a reduction in expression of the three markers, suggesting effects on both chondrogenesis and osteogenesis. MC1568 had no effect on chondrocyte markers and mildly inhibited Col1a1 expression at 6 days. Sirtinol had no effect on Col2a1 expression or chondrocyte differentiation 1 day following exposure; however, it caused a drastic regression in limb cartilage and inhibited the expression of all three markers to nearly undetectable level at 6 days. MS275 and sirtinol caused a 2.2 and 2.7-fold increase in cleaved-caspase 3, a marker of apoptosis, suggesting embryotoxicity.

These data indicate that inhibition of class I and III HDACs causes severe developmental toxicity and is highly teratogenic.

Keywords: HDAC inhibitor, MS275, MC1568, Sirtinol, VPA, teratogen, limb development, chondrogenesis, osteogenesis

4.2 Introduction

Histone deacetylases (HDACs) are enzymes that remove acetyl groups from the lysine residues of histone and non-histone proteins, leading to effects on both gene expression and cellular signaling. HDACs are divided into different classes; class I, II and IV comprise HDAC1-11 whereas class III are SIRT1-7. Inhibitors of class I and II HDACs, such as valproic acid (VPA), an anticonvulsant, and vorinostat (SAHA), an anticancer agent used in the treatment of cutaneous T-cell lymphoma, are used as therapeutics ((Duvic and Vu 2007)). Several other HDACi are currently in development and clinical trials as anticancer agents (Reviewed in (West and Johnstone 2014)); studies suggest HDAC class III inhibitors may also have beneficial effects in the treatment of Parkinson's disease (Outeiro, Kontopoulos et al. 2007, West and Johnstone 2014). However, some HDACi are developmental toxicants in animal models and the embryotoxicity of others is not known (Giavini and Menegola 2014). The specific mechanisms by which HDACi cause malformations remain unresolved (Paradis and Hales, 2014, Murko, Lagger et al. 2013). As new therapeutic indications develop, an increasing number of women of childbearing age may be exposed to HDAC

inhibitors. It is pivotal to decipher the specific roles of individual HDAC classes during development in order to predict the effects of their inhibition.

HDACs are divided into four classes according to the homology of their catalytic domain, structure and cofactor requirements (Taunton, Hassig et al. 1996, Blander and Guarente 2004). HDAC1, 2, 3, and 8 are members of class I and homologous to the yeast Rpd3 protein, whereas HDAC4, 5, 6, 7, 9, 10 are class II HDACs, homologous to Hda1(Yang and Seto 2008). HDAC11 is the only member of class IV and has mixed properties, from class I and II. While classes I, II and IV are all Zn-dependent enzymes, class III HDACs are NAD+-dependent and are commonly referred to as Sirtuins (SIRT1-7), homologous to the yeast Sir2 (Thiagalingam, Cheng et al. 2003). These different enzymes have common targets but they also have distinct high affinity substrates; HDAC1 and SIRT1 have a higher affinity for histones whereas HDAC6 and SIRT2 target alpha-tubulin (Villalba and Alcain 2012). Mouse knockouts for HDAC1, 3 and 7 are embryonic lethal early during organogenesis; the knockouts of several others, such as HDAC2, SIRT1 and 6, are lethal perinatally, suggesting an important role for these enzymes during embryogenesis (Chang, Young et al. 2006, Montgomery, Davis et al. 2007, Montgomery, Potthoff et al. 2008, Finkel, Deng et al. 2009). However, little is known about the specific roles of HDAC classes during development.

The limb has been used as a model system to study organogenesis for many years. The vertebrate skeleton is formed through a process called endochondral ossification and requires the formation of a cartilage template that is subsequently replaced by bone matrix. The cartilage matrix is made of collagen type 2a1 (Col2a1) and is directly regulated by the transcription factor Sox9, expressed in proliferative chondrocytes (Bell,

Leung et al. 1997). These cells differentiate into hypertrophic chondrocytes, expressing the transcription factor Runx2 and the structural protein collagen type 10a1 (Col10a1) (Ding, Lu et al. 2012). Ultimately, these cells undergo cell death or differentiate into osteoblasts that secrete the bone matrix protein collagen type 1a1 (Col1a1) (Karsenty and Park 1995). In a previous study, our group has shown that VPA, a known human teratogen and inhibitor of class I and II HDACs, caused a decrease in Sox9, Col2a1, Runx2 and Col10a1gene expression (Paradis and Hales 2013). Studies suggest that HDAC signaling intervenes in different steps of bone formation; HDAC1 modulates Nkx3.2 and Bmp signaling pathways, both known to play an important role during chondrogenesis (Cairns, Liu et al. 2012, Rigueur, Brugger et al. 2014). HDAC1, 3, 4, 5, 6, and 7 interact with Runx2 (Bradley, McGee-Lawrence et al. 2011). Moreover, HDAC4 knockout mice exhibit premature chondrocyte hypertrophy in the growth plate while mice lacking HDAC2 exhibit a runted phenotype, altogether suggesting congenital bone defects (Vega, Matsuda et al. 2004, Montgomery, Davis et al. 2007).

In this study, we investigated the effects of class-specific HDAC inhibitors on limb development in an in vitro limb bud culture system using class-specific HDAC inhibitors; MS275 (entinostat) inhibits HDAC1 and 3 (Class I), MC1568 inhibits HDAC4 and 6 (Class II) and Sirtinol inhibits SIRT1 and 2 (Class III) (Mai, Massa et al. 2005, Beckers, Burkhardt et al. 2007, Chiara, Ilaria et al. 2014). VPA inhibits both Class I and class II HDACs (Phiel, Zhang et al. 2001). We used triple transgenic mice expressing fluorescently-tagged chondrogenic and osteogenic markers in order to readily assess the effects of our compounds on chondrogenesis and osteogenesis.

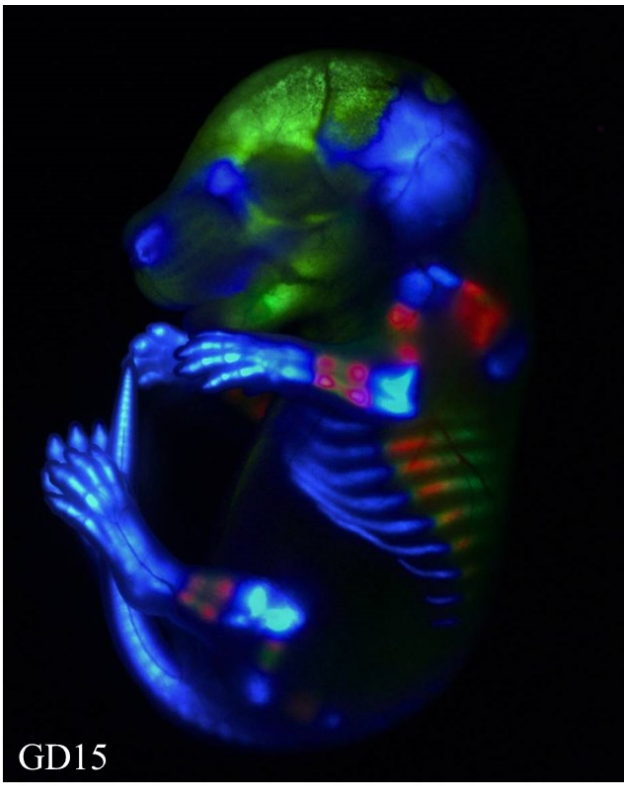
4.3 Material and Methods

Limb bud cultures and drug treatments

Col2a1-eCFP, *Col10a1*-mCherry and *Col1a1*-YFP CD1 reporter mice were a gift from David L. Butler (University of Cincinnati, Cincinnati, OH) and David Rowe (University of Connecticut Health Center, Farmington, CT) (Maye, Fu et al. 2011). These mice express fluorescently-tagged markers of cartilage and bone differentiation (Fig. 4.1). Mice were housed in the McIntyre Animal Resource Centre (McGill University, Montreal, QC, Canada), maintained on a twelve-hour light/dark cycle and allowed access to food and water *ad libitum*. The mice were mated overnight and detection of a vaginal plug was considered gestation day 0 (Magdaleno, Jensen et al. 2006). Timed-pregnant mice were euthanized by cervical dislocation on GD12 and their embryos were explanted. The embryonic forelimbs were cultured, as previously described (Paradis, Huang et al. 2012). Briefly, limbs were excised in Hank's balanced salt solution, pooled and cultured in vitro in 6 ml culture medium consisting of 75% BGJb medium (GIBCO BRL Products, Burlington, ON, Canada) and 25% salt solution supplemented with ascorbic acid (160 µg/ml), and gentamycin (1 µl/ml, GIBCO BRL Products). Each culture was gassed with 50% O_2, 5% CO_2 and 45% N_2 and designated treatments were added; vehicle, sodium valproate (VPA, 3.6 mM, Sigma Chemical Co., St-Louis, MO) dissolved in distilled water, or MS275 (entinostat, 2.5 µM, SelleckChem, Houston, TX), MC1568 (2.5 µM, SelleckChem) or sirtinol (50 µM, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) dissolved in DMSO. Concentrations were chosen in accordance with previous studies and the manufacturers' IC_{50} recommendations

Figure 4.1 Triple transgenic reporter mouse embryo.

Timed-pregnant mice were euthanized and embryos were explanted on gestation day 15 and photographed using a Leica fluorescence stereomicroscope. Proliferative chondrocytes express the Collagen 2a1 tagged with cyan fluorescent protein (Col2a1-CFP) and are shown by blue fluorescence. Hypertrophic chondrocytes express Collagen 10a1 tagged with mCherry (Col10a1-mCherry) and are shown by red fluorescence. Osteoblasts express Collagen 1a1 tagged with yellow fluorescent protein (Col1a1-YFP) and are shown in yellow-green. The use of triple fluorescent tags allows us to readily observe the developing skeleton over time.



(Poljak, Lim et al. 2014). All animal studies complied with the guidelines established by the Canadian Council on Animal Care under McGill University protocol 1825.

Limb morphology and differentiation

Limbs (n=5 cultures, 5-6 limbs per culture bottle) were cultured for 6 days with a change of medium on day 3. On days 1, 3, and 6 of culture pictures were taken using a Leica DFC450C digital camera (Leica Microsystems, Wetzlar, Germany) connected to a Leica M165 Fluorescent Stereo Microscope (Leica Microsystems). Col2a1-CFP positive cartilage was quantified using a morphogenetic scoring system adapted from Neubert and Barrach (Neubert and Barrach 1977). The proportion of limbs expressing the differentiation markers Col10a1-mCherry and Col1a1-YFP were quantified at 3 and 6 days.

Western blotting

Whole cell lysates were obtained by sonication in lysis buffer containing protease inhibitors, consisting of 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5, 40 µg/ml bestatin, 0.2 M phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 6 µg/ml aprotinin. Total protein was extracted and quantified using spectrophotometric Bio-Rad protein assays (Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins (15-30 µg per sample) were separated by SDS-PAGE acrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (BioSciences Inc., Baie d'Urfé, QC, Canada). Precision standards (Bio-Rad Laboratories) were used as molecular weight markers. Membranes were blocked in 5% non-fat milk in TBS-T (137 mM NaC1, 20 mM Tris pH 7.4, 0.05% Tween 20) for 1 h at

room temperature, probed overnight at 4°C with primary antibodies, washed and incubated with the secondary antibody for 2 h at room temperature. Immunoblotting was done using polyclonal antibodies against histone 4 acetyl-lys 12 (H4K12Ac, EMD Millipore, Billerica, MA, 1:5000), alpha-tubulin acetyl-lys40 (Cell Signaling, Danvers, MA, USA, 1:5000), cleaved-caspase 3 (Cell Signaling, 1:1000), hdac1 (Cell Signaling, 1:2500), and beta-actin (Santa Cruz Biotechnology, Inc., 1:10000). Monoclonal antibodies against sirt1 (Abcam, Cambridge, MA, 1:2500) and hdac6 (Abcam, 1:2500) were used. The secondary antibodies, conjugated to horseradish peroxidase (HRP), were anti-rabbit (GE Healthcare Limited, Baie d'Urfé, QC, Canada, 1:5000), anti-mouse (GE Healthcare Limited, 1:5000) and anti-goat antibodies (Santa Cruz Biotechnology, Inc, 1:10000). Western blots were visualized with the Enhanced Chemiluminescence Plus Kit (GE Healthcare Limited) and quantified by densitometry using a ImageJ imaging software (NIH, Bethesda, MD, USA).

Statistical Analyses

All data sets were analyzed statistically using SigmaPlot 11.0 (Systat Software, San Jose, CA, USA). The Ranked Mann-Whitney U test and multiple-comparisons were used, with Bonferonni's correction. The minimum level of significance was $P < 0.05$.

4.4 Results

MS275 and MC1568 exposures induce hyperacetylation of histone h4 and tubulin

To examine the HDAC inhibition activity of our class-specific inhibitors, we used histone4 acetylation as a marker of the activity of nuclear HDACs and tubulin acetylation as a marker of activity in the cytoplasm (Fig. 4.2). MS275, an inhibitor of HDAC1 and 3, induced a significant increase in H4k12Ac whereas MC1568, an inhibitor of HDAC4 and 6, caused a hyperacetylation of tubulin. Surprisingly, sirtinol, an inhibitor of SIRT1 and 2, did not cause an increase in acetylation of either target. We confirmed the expression of these targets in the developing limb by western blot (Figure S4.1). Together, these data suggest that SIRT1 and SIRT2 have a low affinity for these targets in the developing limb than in other cell types. The HDAC inhibition activity of VPA in the limb bud culture model was characterized in a previous study.

Class-specific HDAC inhibitors have distinct effects on chondrogenesis and osteogenesis

To assess the effects of MS275 on chondrogenesis and osteogenesis, triple transgenic forelimbs were cultured in vitro in the presence of MS275 for 6 days. MS275 induced a decrease in CFP fluorescence (44% decrease in limb score) at 24h following exposure; changes were also observed at 3 and 6 days (76% and 66% decrease in score) (Fig. 4.3). Phalanges were often missing and metacarpals were underdeveloped, short and round. Hypertrophic chondrocytes and osteoblasts failed to differentiate, as shown by the complete absence of both mCherry and YFP fluorescence.

Figure 4.2 HDACs are inhibited following exposure to MC1568 or MS275.

Forelimbs were cultured for 3 or 24h in the presence of vehicle (C, control), 50 μM sirtinol (S), 2.5 μM MC1568 (MC) or 2.5 μM MS275 (MS). Whole cell lysates were extracted, proteins were separated by electrophoresis, transferred onto a PVDF membrane and detected by immunoblotting (A). Actin was used as a loading control. Bands were quantified by densitometry (B, C). * = $p<0.05$, n=5-6.

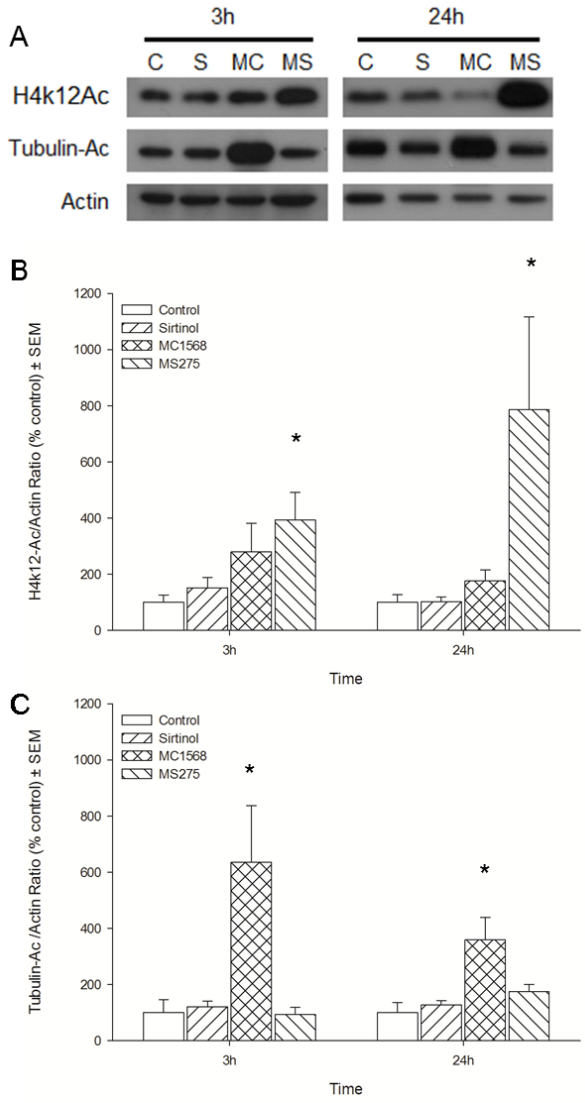
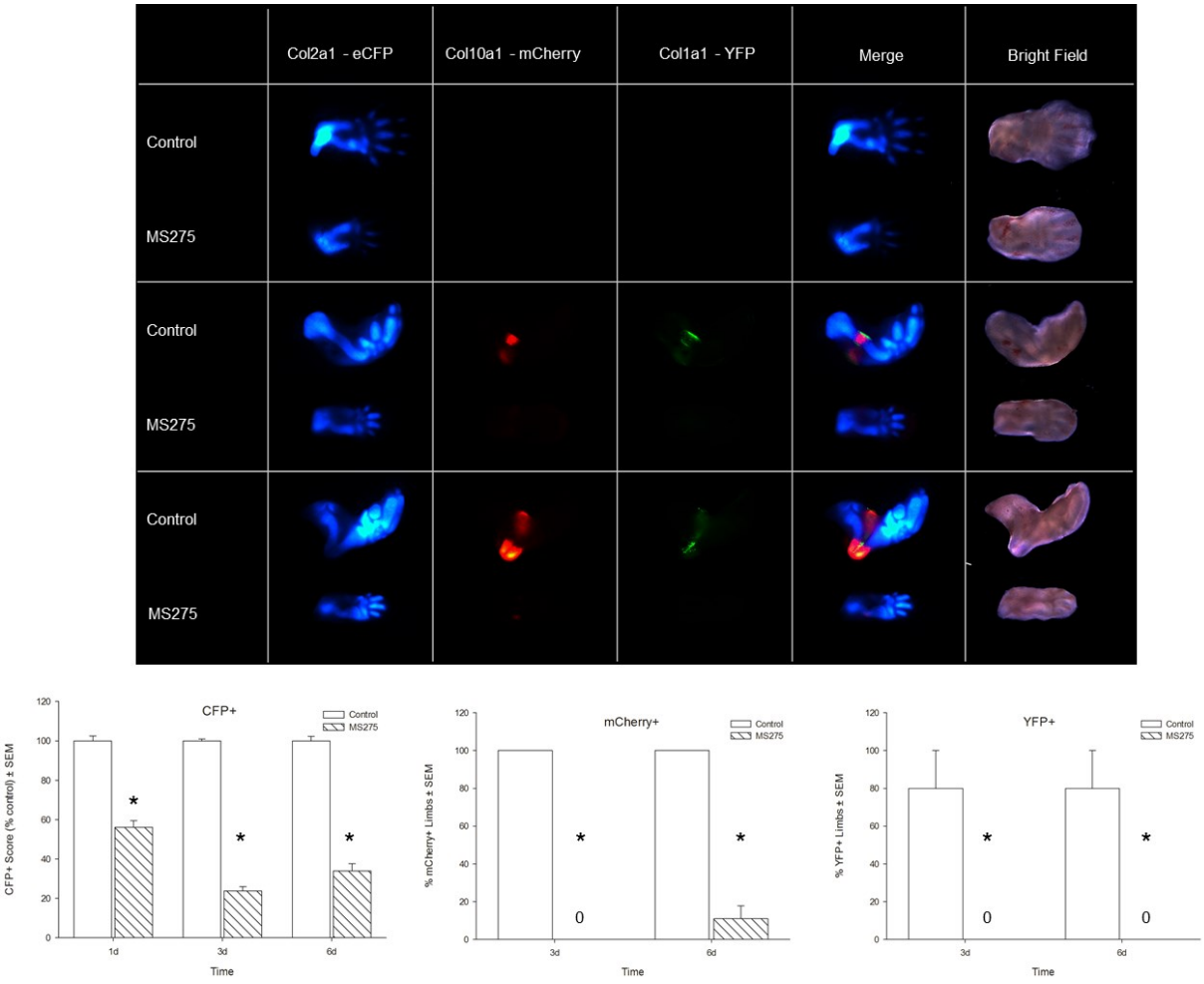


Figure 4.3 Chondrogenesis and osteogenesis were decreased following inhibition of Class I HDACs.

GD12 forelimbs were cultured in presence of vehicle or 2.5 μM MS275 for 6 days. Pictures were taken on days 1, 3 and 6 (upper panel). CFP positive cartilage was scored according to its differentiation; each component of the limb - the digits, the carpalia, the ulna and the radius – was given a score from 0 when completely absent to 30 when perfectly differentiated. Quantification of the percent occurrence of mCherry and YFP positive limbs is depicted in the lower panel. *= $p<0.05$, n=5.



In contrast, MC1568 treatment had no effects on chondrogenesis or on CFP positive score and only a mild non-significant effect on mCherry fluorescence at 3 days (Fig.4.4). A decrease in Col1a1-YFP was observed at 6 days, suggesting that inhibition of HDAC class II affects osteoblast differentiation.

Sirtinol exposure led to a phenotype distinct from that of either MS275 or MC1568 (Fig.4.5). There were no changes in CFP positive scores at 1 day and drastic decreases, to 42 and 91%, at 3 and 6 days, respectively, in sirtinol-treated limbs. Col10a1-mCherry and Col1a1-YFP fluorescence were also reduced at both 3 and 6 days. Moreover, qualitative analysis of the sirtinol-treated limbs revealed radical effects on limb morphology, suggesting severe cytotoxicity (Fig.4.5).

The effects of VPA on chondrogenesis and osteogenesis are shown in Fig.4.6. VPA treatment had very similar effect to that of MS275; several digital condensations were missing and a rapid decrease in CFP positive score was observed at 1, 3 and 6 days following exposure (50, 61 and 60% decrease in limb score respectively); the numbers of limbs exhibiting the differentiation markers Col10a1-mCherry and Col1a1-YFP were significantly reduced at both 3 and 6 days.

MS275 and sirtinol induce activation of caspase 3

Using cleaved-caspase3 as a marker our next goal was to assess the extent of apoptosis in treated limbs (Fig.4.7). MS275 and sirtinol both significantly increased the cleavage of caspase 3 at 24 h following exposure, suggesting an increase in cellular death consistent with the limb phenotype we observed; in contrast, MC1568 did not

Figure 4.4 Chondrogenesis and osteogenesis were unaffected by exposure to MC1568.

GD12 forelimbs were cultured in presence of vehicle or 2.5 μM MC1568 for 6 days. Pictures were taken on day 1, 3 and 6 (upper panel). CFP positive cartilage was scored according to its differentiation; each component of the limb - the digits, the carpalia, the ulna and the radius – was given a score from 0 when completely absent to 30 when perfectly differentiated. Quantification of the percent occurrence of mCherry and YFP positive limbs is depicted in the lower panel. *= $p<0.05$, n=5.

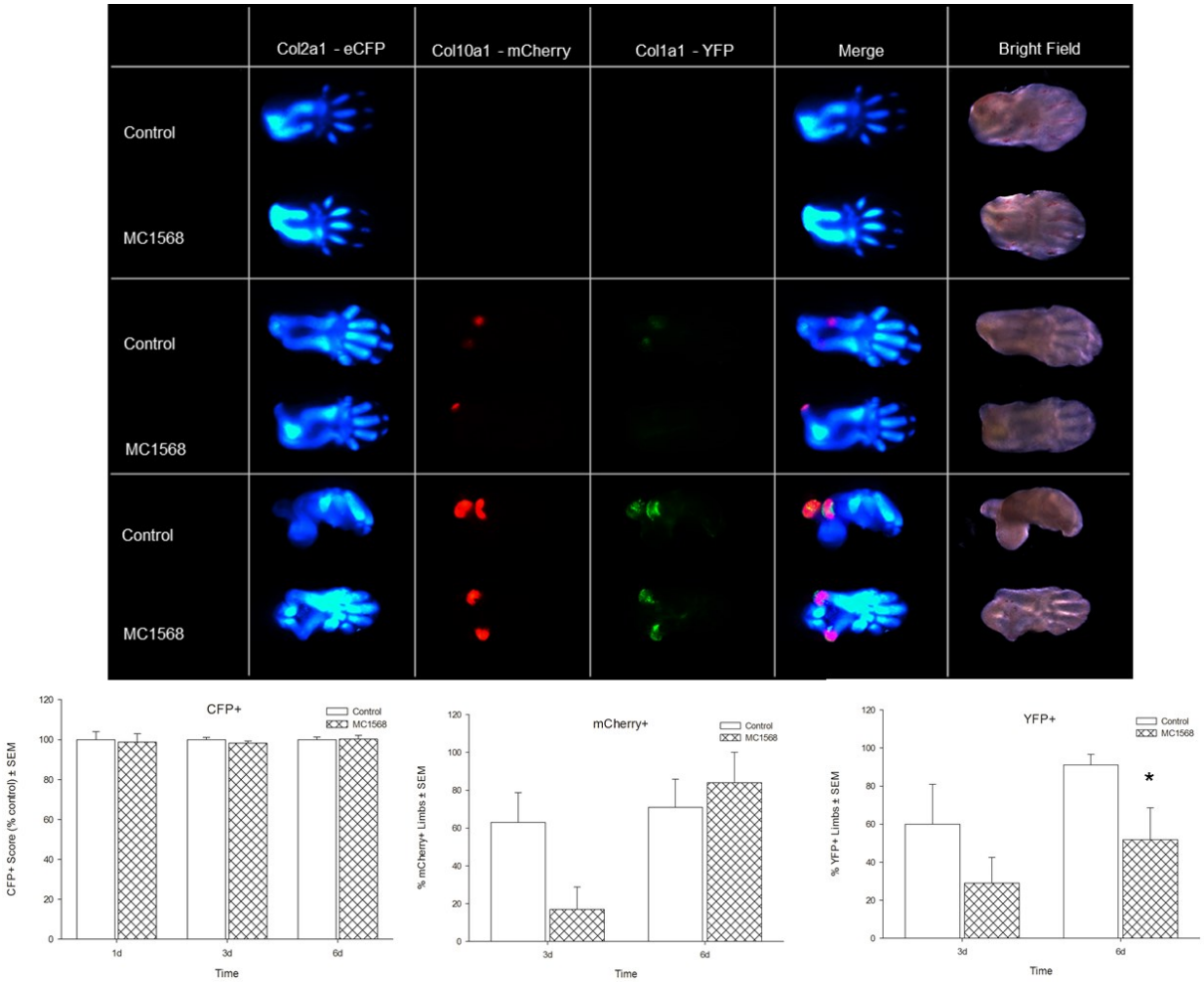


Figure 4.5 Sirtinol had drastic effects on limb morphology.

GD12 forelimbs were cultured in presence of vehicle or 50 μM sirtinol for 6 days. Pictures were taken on days 1, 3 and 6 (upper panel). CFP positive cartilage was scored according to its differentiation; each component of the limb - the digits, the carpalia, the ulna and the radius – was given a score from 0 when completely absent to 30 when perfectly differentiated. Quantification of the percent occurrence of mCherry and YFP+ limbs is depicted in the lower panel. *= p<0.05, n=5.

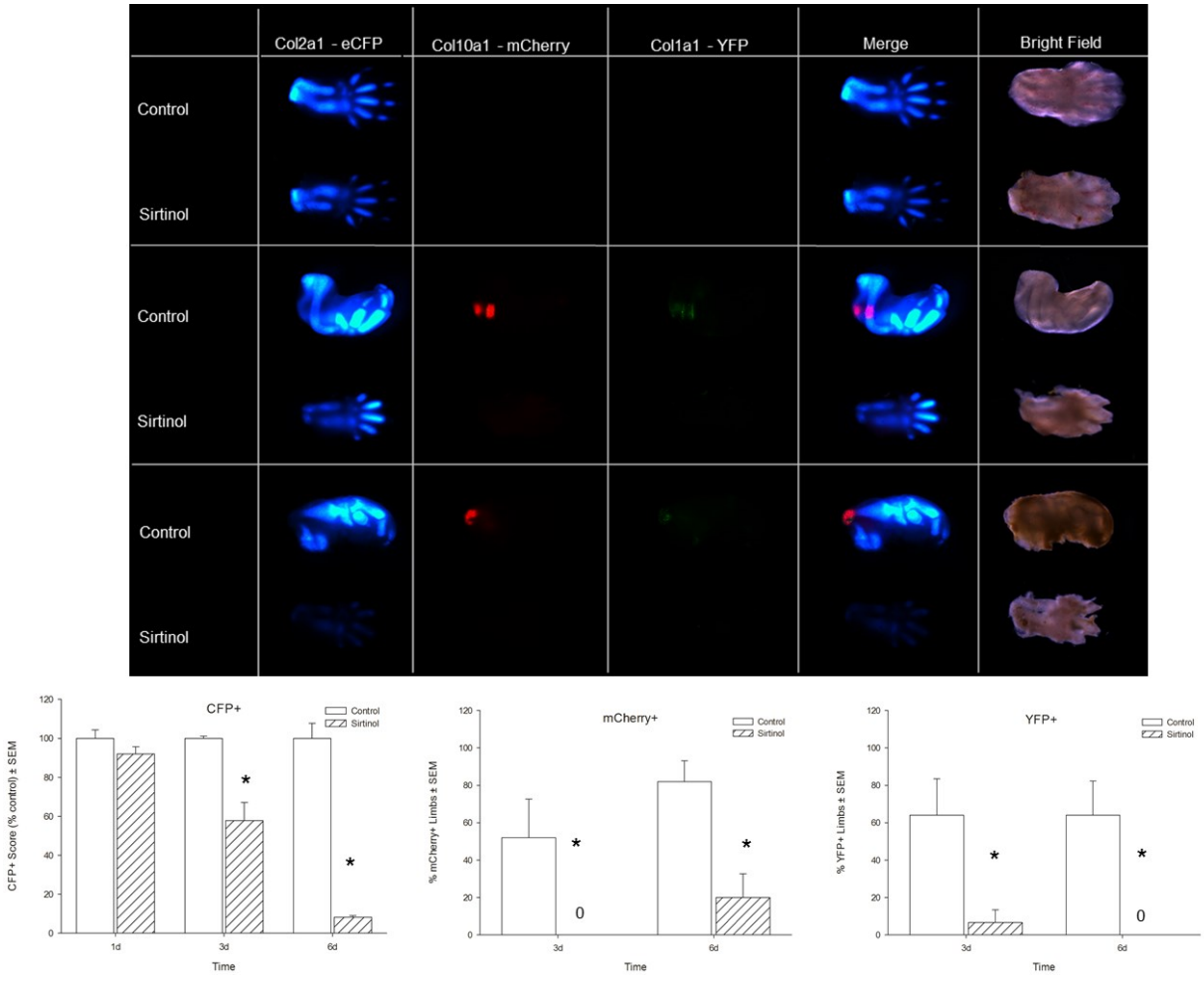


Figure 4.6 VPA inhibits chondrogenesis and osteogenesis.

GD12 forelimbs were cultured in presence of vehicle or 3.6 mM VPA for 6 days. Pictures were taken on days 1, 3 and 6 (upper panel). CFP positive cartilage was scored according to its differentiation; each component of the limb - the digits, the carpalia, the ulna and the radius – was given a score from 0 when completely absent to 30 when perfectly differentiated. Quantification of the percent occurrence of mCherry and YFP positive limbs is depicted in the lower panel. *= $p<0.05$, n=5.

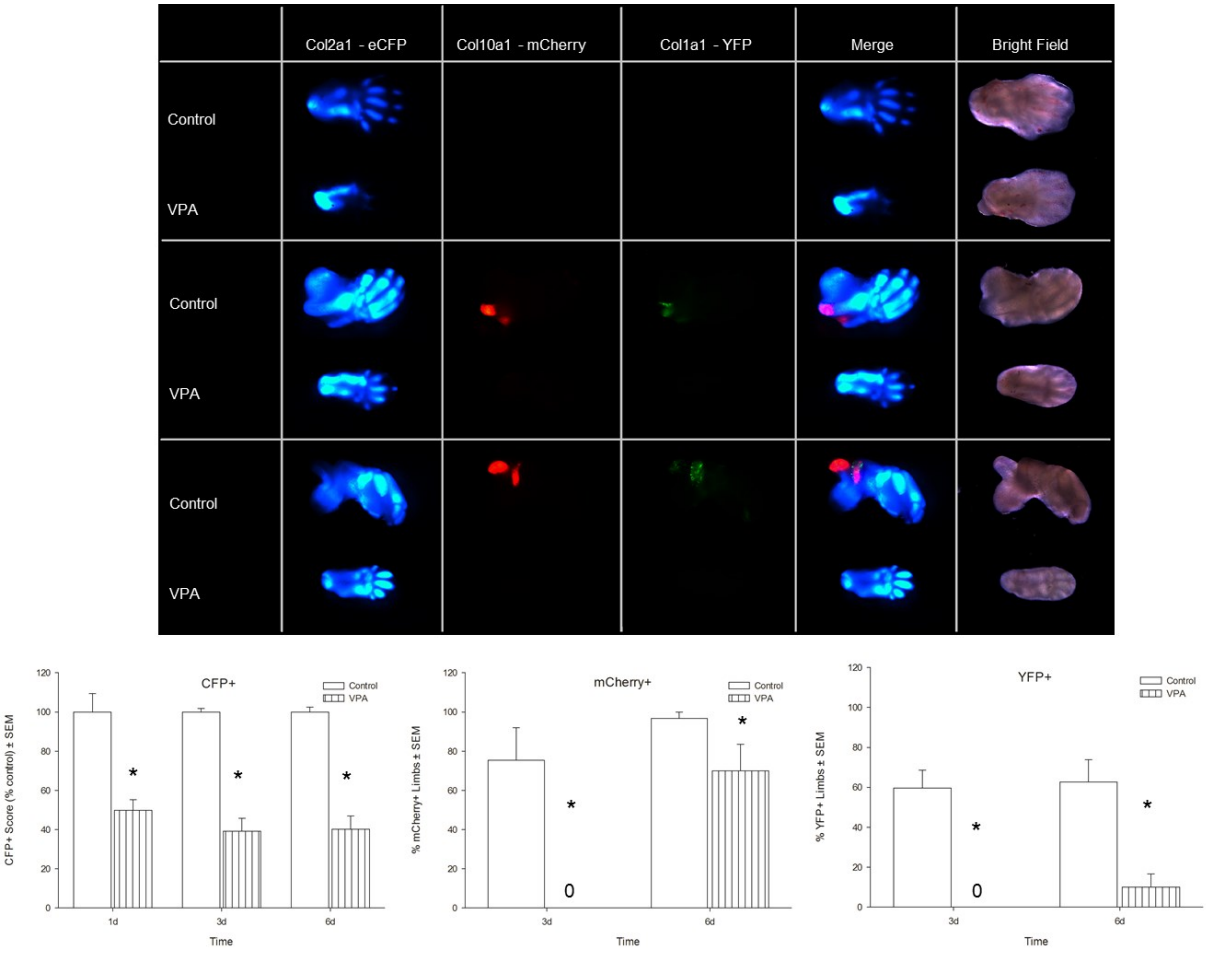
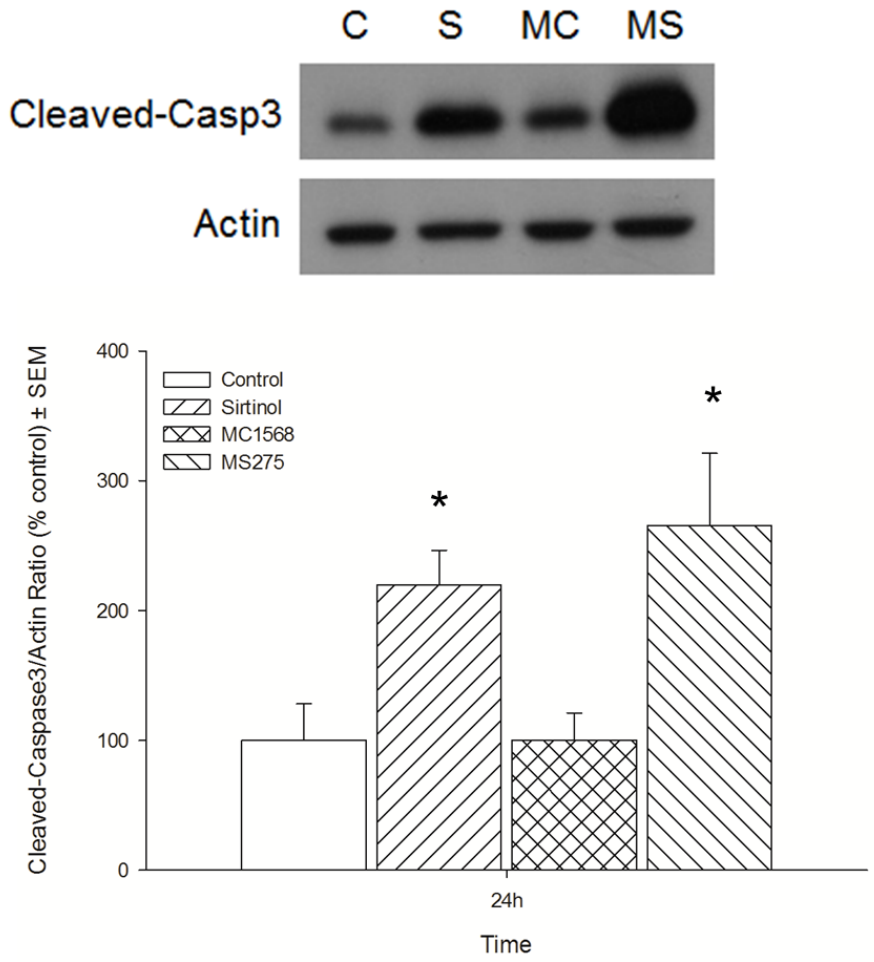


Figure 4.7 Inhibition of HDAC classes I and III triggered cellular apoptosis.

Cultures were stopped after 24h and whole cell lysates were extracted. Proteins were separated by electrophoresis, transferred onto a PVDF membrane and antibodies against cleaved-caspase 3 were used to detect the protein expression (Upper panel). Actin was used as a loading control. Bands were quantified by densitometry (Lower panel). * = $p<0.05$, n =5.



increase cleaved caspase 3, suggesting that class II inhibition is not toxic to the cells. VPA-induced caspase 3 activation in the limb bud culture model was characterized in another study (chapter 3).

4.5 Discussion

Class-specific HDAC inhibitors had distinct effects on limb development. Inhibition of class I or III HDACs had highly detrimental effects on skeletal development and was cytotoxic, whereas inhibition of class II HDACs had only mild effects on bone progenitor differentiation. To our knowledge, this is the first evidence that sirtinol, an HDAC class III inhibitor, is embryotoxic during organogenesis.

The limb skeleton develops through the same process as other long bones such as the ribs and vertebrae, via endochondral ossification. The flat bones of the body, including the bones of the skull, pelvis, scapula, and mandible, are formed by intramembranous ossification. Both processes require *Runx2* and *Col10a1* expression, suggesting that the formation of the entire skeleton could be affected by the inhibition of this pathway.

MS275 and sirtinol both triggered programmed cell death in the developing limb. Teratogen-induced apoptosis in embryonic tissue has been associated with several birth defects. VPA-induced apoptosis in the somites on gestational day 9 has been associated with an increase in neural tube defects (Di Renzo, Broccia et al. 2010). The severity of the malformations induced by treatment with hydroxyurea, an anticancer drug, and the organs targeted were correlated with an increase in apoptosis and DNA fragmentation in specific tissues (Banh and Hales 2013, Schlisser and Hales 2013).

Thus, the enhanced apoptosis induced by HDAC class I and III inhibition in our limb model may also be observed in other malformation-sensitive tissues and organs during organogenesis. However, further studies are needed to investigate the molecular signalling pathways leading to this increase in cellular death.

The VPA-induced rapid decrease in Col2a1 expression and decrease in mesenchymal condensation were remarkably similar to those observed with MS275, suggesting these are mediated through inhibition of HDAC class I, rather than class II. In a previous study, we showed that VPA exposure induced a rapid decrease in Sox9 and Runx2 gene expression; these effects were correlated with HDAC inhibition since a non-HDACi analog of VPA, valpromide, was inactive (Paradis and Hales, 2013). Whether Sox9 and Runx2 signaling pathways are affected by our drug treatments remains to be investigated.

MS275, MC1568 and sirtinol target HDAC1/3, HDAC4/6 and SIRT1/2, respectively. HDAC1 knockout mice are embryonic lethal prior to limb bud formation (Montgomery, Davis et al. 2007). In vitro, HDAC1 is involved in a number of pathways that regulate chondrogenesis, such as *Nkx3.2* (Cairns, Liu et al. 2012). HDAC3 knockout mice are runted, have fewer osteoblasts than their wildtype littermates and exhibit a decrease in bone density (Razidlo, Whitney et al. 2010). These data are consistent with our results, suggesting these members of the class I HDACs play pivotal roles during limb differentiation.

The absence of effect on chondrogenesis and the mild effects on osteogenesis that we observe in MC1568-exposed limbs are inconsistent with the phenotype observed in HDAC4 knockout mice that exhibits reduced cartilage formation, premature chondrocyte

hypertrophy and ossification. In contrast, MC1568 inhibits matrix metalloproteinase (MMP) *9* gene expression in extraembryonic tissue primary cell cultures (Poljak, Lim et al. 2014). MMP9 is a matrix metalloproteinase that is essential for endochondral ossification; these metalloproteinases degrade the cartilage extracellular matrix in order to create gaps where the osteoblasts will deposit the bone matrix (Ortega, Behonick et al. 2003). MC1568 may interfere with this degradation process in our model, thereby hindering osteoblast differentiation and *Col1a1* expression. More studies are needed to assess if the MC1568-triggered delay in ossification is transient or permanent.

Our results are consistent with the phenotype observed in SIRT1 knockout mice; both their axial and appendicular skeletons exhibit abnormalities although these are not as severe as the ones we observed in this study (Gabay, Zaal et al. 2013). This suggests that SIRT1 and SIRT2 have complementary effects. Alternatively, *Sirt1*-/- mice may have compensatory mechanisms with the consequence that a pharmacologically-induced transient inhibition of SIRT1 has more a detrimental effect.

Altogether, this study provides valuable evidence for the diverse teratogenic effects of three class-specific HDAC inhibitors on mouse embryonic skeletal development. We showed that class I and III inhibitors are highly teratogenic and cause drastic structural malformations, whereas a class II inhibitor has only minor effects on ossification. Identifying the molecular pathways and targets leading to teratogenesis is pivotal in order to rapidly identify potential developmental toxicant and prevent their effects.

4.6 Supplementary Data description

Supplementary figures are provided for the protein expression analysis of HDAC1, HDAC6 and SIRT1, the molecular targets of the pharmacological inhibitors.

4.7 Funding Information

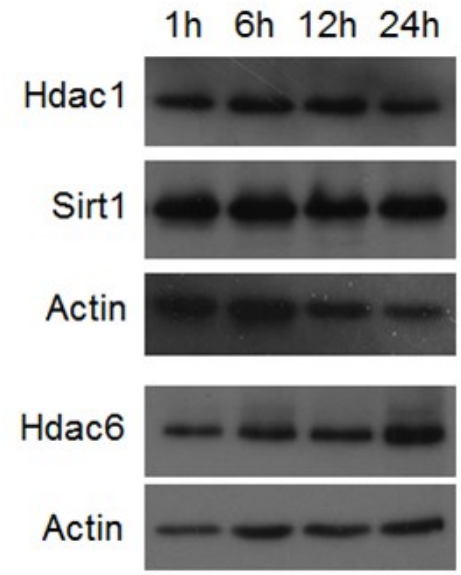
These studies were supported by grants from CIHR awarded to BFH and an FRQS fellowship awarded to FP. BFH is a James McGill Professor.

4.8 Acknowledgements

We thank Dr. David L. Butler (University of Cincinnati, Cincinnati, OH) and Dr. David Rowe (University of Connecticut Health Center, Farmington, CT) for providing the Col2a1-ECFP, Col10a1-mCherry and Col1a1-YFP reporter mice.

Supplementary Figure S 4.1 Drug targets were expressed in GD12 forelimbs.

Control cultures were stopped after 1, 6 12 or 24h and whole cell lysates were extracted. Proteins were separated by electrophoresis and proteins were detected by immunoblotting n=2.



Chapter 5 Discussion

5.1 Summary

The central hypothesis of this thesis was that VPA exposure inhibits HDACs and affects signaling pathways that are crucial for processes involved in organogenesis, ultimately leading to teratogenic effects. The studies presented herein addressed this hypothesis by investigating the changes in HDAC activity, cellular differentiation and the apoptotic response of cells in the developing limb bud following exposure to VPA. Specifically, in an in vitro limb bud culture system, VPA caused a concentration-dependant inhibition of HDACs and a downregulation of both Sox9 and Runx2 signaling pathways, two pivotal regulators of chondrogenesis and osteogenesis (Chapter 2). In addition, p53 signaling and apoptosis were triggered following VPA exposure (Chapter 3). The effects observed in VPA-treated limbs in chapter 2 and 3 were not seen in VPD-treated limb suggesting an HDAC dependent mechanism. To further investigate the role of HDACs in limb development, class-specific HDAC inhibitors were studied (Chapter 4). Inhibition of class I HDACs caused detrimental effects on limb morphology, chondrocyte and osteocyte differentiation as well as on apoptosis; in contrast, inhibition of class II HDACs caused minor detrimental effects. Inhibition of class III HDACs did not interfere with the initiation of differentiation but caused drastic effects on cellular survival and highly diminished organ health. These findings support the hypothesis that VPA causes teratogenic effects through the inhibition of a specific HDAC class, namely HDAC class I.

5.2 Linking Sox9 signaling and chondrogenesis to HDAC signalling

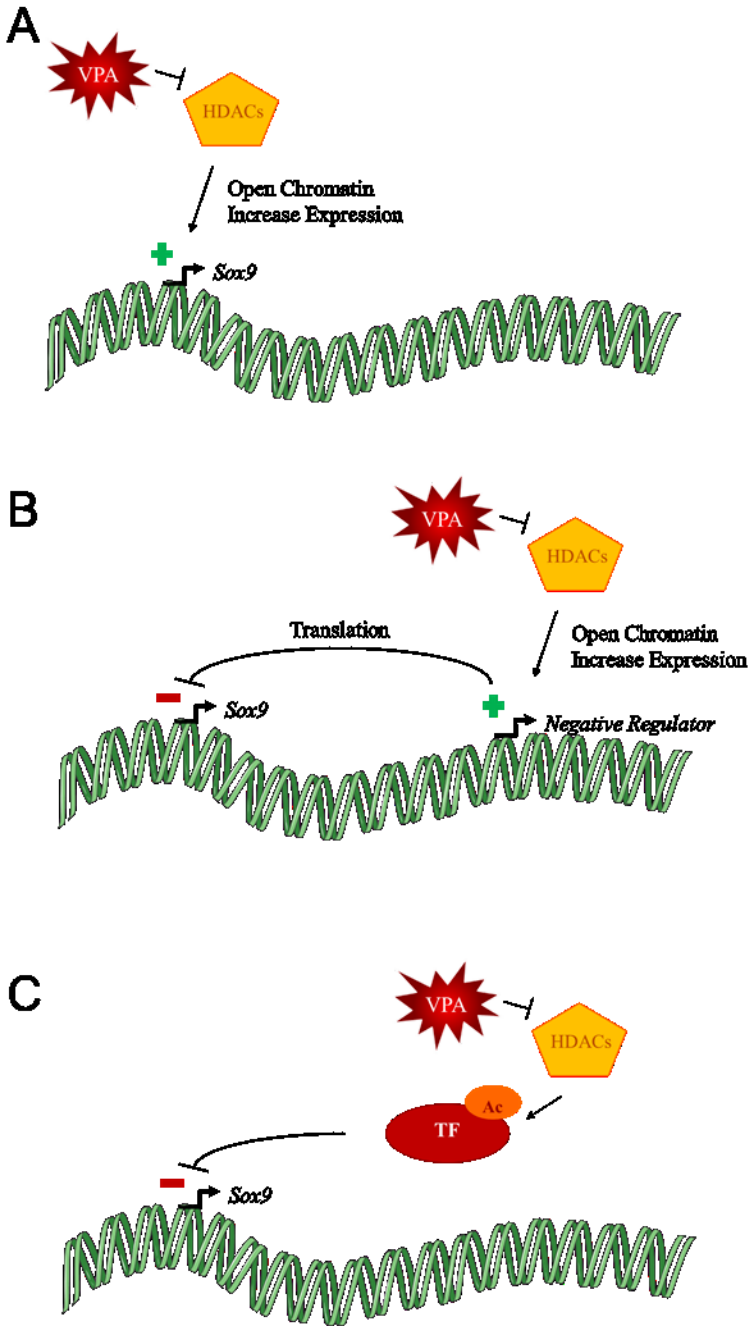
Studies presented in Chapter 2 of this thesis show a correlation between HDAC inhibition and reduced chondrogenesis and osteogenesis using VPD as a comparative tool. As mentioned previously, both SOX9 and RUNX2 interact with HDACs and are acetylated. However, their acetylation leads to an increase in their transcriptional activation that is inconsistent with the decrease in mRNA levels of their downstream targets (Jeon, Lee et al. 2006, Hattori, Coustry et al. 2008).

Exposure to actinomycin D blocked VPA-induced downregulation of sox9 mRNA, suggesting this rapid effect is transcription dependant and is not achieved through an increase in mRNA degradation (Chapter 2). We propose three scenarios by which inhibition of HDAC would affect the transcription of sox9 that we observed. First, a direct action of HDAC inhibition on chromatin would result in histone hyperacetylation, open chromatin and increased gene expression (Fig.5.1A). However, we observed a repression of sox9 transcription which contradicts scenario 1. An indirect effect leading to the upregulation of a transcriptional regulator of sox9 promoter would require de novo translation (Fig.5.1B). This scenario is unlikely as the effect on sox9 transcription was observed at one hour following exposure. Finally, the third scenario would involve a direct interaction between HDACs and transcriptional regulators of the sox9 promoter (Fig.5.1C). This last scenario is our current working hypothesis. Analysis of the Sox9 promoter revealed binding sites for several transcription factors that are known to be HDAC targets.

NF-kB binds directly to the promoter of sox9 and its activity is modulated by its

Figure 5.1 Proposed scenarios of HDAC inhibition-mediated transcriptional dysregulation.

A. A direct effect of HDAC on the histone tail and the chromatin surrounding the gene promoter region leading to open chromatin and an increase in transcription. B. An indirect effect of HDAC on the gene expression through a direct effect on the chromatin surrounding the gene promoter region of a transcriptional regulator. This would lead to an opening of the chromatin, increase transcription of the regulator, translation and subsequent modulation of the target genes (i.e *Sox9*). C. A direct effect of HDAC on the acetylation of a non-histone target, namely a transcription factor or modulator, leading to the transcriptional repression of the target gene.


A
VPA
HDACs
Open Chromatin
Increase Expression
Sox9
B
VPA
HDACs
Translation
Open Chromatin
Increase Expression
Sox9
Negative Regulator
C
VPA
HDACs
Ac
TF
Sox9

acetylation at different lysine residues (Greene and Chen 2004, Sun, Uozaki et al. 2012). Mice with a decreased expression of the inhibitor of kappa kinase α (IKKα), a regulator of the NF-kB pathway, have impaired limb outgrowth (Takeda, Takeuchi et al. 1999). On the other hand, members of the BMP family play numerous roles in skeletogenesis (Reviewed in (Li and Cao 2006)). BMP2 exposure induces the expression of *Sox9* and differentiation of mouse embryonic fibroblasts into chondrocytes (Pan, Yu et al. 2008). BMPs are part of the transforming growth factor beta (TGFβ) family whose signaling is mediated by three classes of smads; the receptor-regulated R-SMAD (SMAD1, 2, 3, 5, 8, 9), co-SMAD (SMAD4) and inhibitory smad I-SMADs (SMAD6, 7) (Massague, Seoane et al. 2005). A predicted binding site for SMAD proteins was also found in our analysis. The stability of SMAD7 is regulated by its acetylation; this acetylation competes with ubiquitination of lysine residues (Gronroos, Hellman et al. 2002). Therefore, NF-kB and SMAD7 constitute interesting candidates for further investigations into the mechanism of HDAC-mediated inhibition of *sox9* transcription (Fig.5.2).

5.2.1 Multiple roles of *sox9* regulation in teratogenesis

Sox9 regulation was altered following exposure to other developmental toxicants and plays a role in the development of a number of different organs; *Sox9* mRNA expression was shown to be altered in limbs following exposure to another teratogen, retinoic acid (Galdones, Lohnes et al. 2006). Several derivatives of vitamin A, including retinoic acid, have been shown to be teratogenic and cause limb malformations both in humans and animal models.

In addition to its role in chondrogenesis and limb development, *sox9* plays pivotal roles in testis development (Kanai, Hiramatsu et al. 2005). Conditional homozygous inactivation of *sox9* in the mouse gonadal anlagen leads to lack of testis cord formation and sex reversal (Barrionuevo, Bagheri-Fam et al. 2006). Exposure to VPA and bisphenol A have been associated both with an increased risk of hypospadias and a decrease in *Sox9* mRNA expression (Morrow, Russell et al. 2006, Aoki and Takada 2012, Choi, Kim et al. 2012). Furthermore, a mutation in the *Sox9* gene is linked to a human condition called Pierre-Robin syndrome characterized by congenital cleft palate, suggesting that SOX9 may play a role in craniofacial formation (Selvi and Mukunda Priyanka 2013). As VPA often causes of skeletal, urogenital and craniofacial defects, VPA-induced downregulation of *Sox9* could play an important role in mediating multiple facets of its teratogenesis.

5.3 Linking RUNX2 signaling and osteogenesis to HDACs

VPA exposure caused a downregulation of *Runx2* at 3h following exposure. Differentiated osteoblasts could not be obtained in a primary culture and we were unable to test the mRNA stability of *Runx2* using actinomycin D. Therefore, VPA-induced downregulation could be due to a decrease in stability of the transcript, an indirect effect of HDAC inhibition of the chromatin at a cis promoter (Fig. 5.1B) or a post-translational modification of a transcriptional regulator of Runx2 (Fig. 5.1C). We will therefore discuss these different scenarios.

A group of 11 Runx2-targeting miRNAs, including miR-34c, were shown to be expressed in an inversely proportional manner to *runx2* mRNA and protein level in

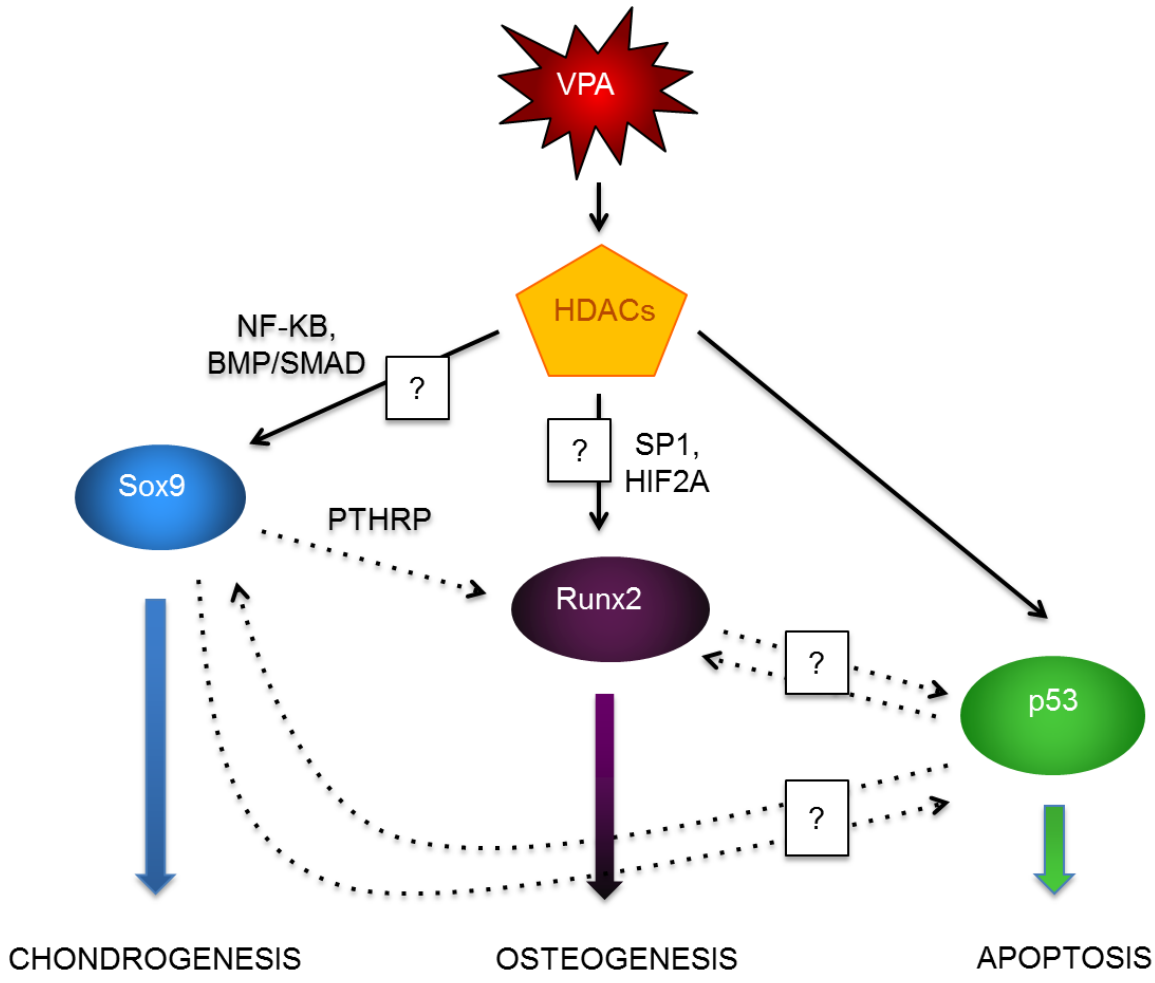
osteoblast cell lines (Zhang, Xie et al. 2011), This suggests that *Runx2* is subject to post-transcriptional and translational regulation. Moreover, VPA exposure increased DICER degradation and thereby affected the miRNA expression (Zhang, Convertini et al. 2013). VPA exposure altered the expression of 110 miRNAs in mouse embryonic stem cells and several of these changes were also observed in trichostatin A-exposed cells, suggesting an HDAC-dependant mechanism (Smirnova, Block et al. 2014).

Several transcriptional regulators of the *Runx2* promoter have been identified; SP1 and hypoxia-inducible factor 2α (HIF2A) directly bind the promoter and increase Runx2 expression (Tamiya, Ikeda et al. 2008, Zhang, Hassan et al. 2009). SP1 and HIF2A have also been shown to interact in hypoxic conditions and form a complex with HDACs (Koizume, Ito et al. 2012). SP1 has a number of posttranslational modifications and was shown to be hyperacetylated following exposure to HDACi (Huang, Zhao et al. 2005). Together these data suggest that SP1 and HIF2A could be interesting candidates to examine in future studies (Fig.5.2).

Furthermore, chondrogenesis and osteogenesis are closely related processes and SOX9 regulates the expression of RUNX2 at multiple levels. SOX9 regulates the expression of parathyroid hormone-related protein (PTHrP), that binds the parathyroid hormone receptor (PTHR) (Akiyama, Chaboissier et al. 2002). PTHR activation leads to a downregulation of *Runx2* (Guo, Chung et al. 2006). SOX9-induced NKX3.2 expression represses *Runx2* expression (Yamashita, Andoh et al. 2009). SOX9 also directly interacts with RUNX2 and causes its degradation (Cheng and Genever 2010). Therefore, the effect of VPA on the RUNX2 pathway could be due to alterations in

Figure 5.2 Schematic representation of interactions between the different pathways investigated in this thesis as well as proposed future avenues of investigation.

Solid lines are effects observed in our data. Dotted lines are links established in the literature.



SOX9 signaling. However, we observed a downregulation of both SOX9 and RUNX2 signaling, suggesting these two effects may be independent.

5.4 Linking SOX9 and RUNX2 to P53

VPA-exposed limbs exhibited effects on SOX9 (chondrogenesis), RUNX2 (osteogenesis) and P53 (apoptosis) (Chapter 2 and 3). Although they have very distinct functions during development, SOX9, RUNX2 and P53 signaling are interconnected at various levels. SOX9 overexpression causes a decrease in P53 protein level whereas P53 activation by Nutlin-3 leads to an overexpression of *Sox9* (Panda, Miao et al. 2001, Lion, Bisio et al. 2013) . Moreover, limb buds lacking *Sox9* expression exhibit an increase in cellular apoptosis (Akiyama, Chaboissier et al. 2002).

RUNX2 binds to the promoter of P53-responsive genes and neutralizes its action, leading to an anti-apoptotic signal. In addition, *Runx2* knock down enhances P53 target gene expression and cellular apoptosis (Ozaki, Wu et al. 2013). Conversely, P53 induces the expression of *miR-34c*, leading to a downregulation of RUNX2; osteoblasts of *p53* null mice exhibit increased RUNX2 expression whereas concentration dependant increase in P53 activation by Nutlin-3 are highly correlated with a decrease in *Runx2* mRNA (van der Deen, Taipaleenmaki et al. 2013).

In our study, *Sox9* mRNA is transiently downregulated from 1 to 6h following VPA exposure whereas P53 signaling is activated at 3h (Chapter 2). These results might suggest a role for P53 in the feedback regulation of *Sox9* mRNA in VPA-exposed chondrocytes. Furthermore, VPA exposure induced both a downregulation of RUNX2 signaling and an activation of P53 signaling at 3h (Chapter 2 and 3). The results

presented in these studies are consistent with an effect of the RUNX2 pathway on P53 signaling and vice versa. However, additional experiments are required to test these hypotheses and elucidate the interaction between these pathways as well as their role in the VPA-exposed developing limb (Fig.5.2).

5.5 The importance of P53 signaling and apoptosis

VPA-exposed limbs exhibited a sequential activation of the P53 signaling pathway prior to the onset of apoptosis (chapter 3). These results are consistent with those presented by Di Renzo et al. showing an increase in P53 acetylation and induction of apoptosis in somites of VPA exposed embryos (Di Renzo, Broccia et al. 2010). However, their study showed an apoptotic cascade independent of caspase 3 induction, suggesting that VPA-induced P53 activation plays a role in caspase-independent apoptosis (Di Renzo, Broccia et al. 2010). On the other hand, oxidative stress and DNA damage are known inducers of P53 signaling and were shown to be induced and involved in VPA-induced cell death (Defoort, Kim et al. 2006); in whole embryo cultures apoptosis in the embryonic neural tube was rescued by co-exposure to exogenous antioxidant catalase enzymes (Tung and Winn 2011). We also observed an increase in a DNA damage marker, phosphorylated histone H2AX, following VPA exposure in the limb (Chapter 3). However, P53 activation occurred prior to the induction of H2AX phosphorylation, suggesting these are activated independently.

Several studies have shown a controversial role for P53 in the response to toxic insult during embryogenesis; *p53* was shown to be protective and decrease 4-hydroperoxycyclophosphamide induced limb malformations (Moallem and Hales 1998).

P53 null mice exhibited higher embryotoxicity following exposure to benzo[a]pyrene than their wildtype littermates (Nicol, Harrison et al. 1995). In contrast, 2-chloro-2-deoxyadenosine-induced eye defects were highly diminished in *p53*-null mutants, suggesting that P53 mediates this effect (Wubah, Ibrahim et al. 1996). Altogether, these data suggest that P53 plays an important role in the response to teratogen exposure although its specific role could be compound specific or tissue specific.

5.6 HDAC class specific effects and the role of class I HDAC inhibition in VPA-mediated teratogenesis

Exposure to VPA and MS275 had remarkably similar effects on chondrocyte and osteoblast differentiation as assessed by the expression of COL2A1 and COL10A1 (Chapter 4). These results, together with the mild teratogenicity exhibited in MC1568-exposed limbs, suggest that class I HDACs may be the mediators of VPA-induced teratogenicity. Although SIRT1 was shown to promote chondrocyte differentiation in vitro, its inhibition by sirtinol did not affect the initiation of chondrogenesis and the formation of the mesenchymal condensation in our model system (Chapter 4) (Buhrmann, Busch et al. 2014). Sirtinol-induced reduction in this chondrogenesis marker was a late response that could involve collagen degrading enzymes such as matrix metalloproteinase (MMPs). Inhibition of SIRT1 has been associated with an increase in MMP9 and MMP13 expression (Nakamaru, Vuppusetty et al. 2009, Fei, Shimizu et al. 2015).

Increased cellular apoptosis was observed following exposure to VPA, MS275 and sirtinol, but not MC1568 or VPD. This shows a direct correlation between the

morphological differentiation of the limb and the induction of cellular death (chapter 3, 4). HDAC1 and SIRT1 have been shown to directly interact with p53, causing its acetylation, suggesting that P53 signaling may be involved in MS275 and sirtinol-induced teratogenesis (Ito, Kawaguchi et al. 2002, Li, Wang et al. 2012). In contrast, MC1568 does not increase P53 acetylation (Mellert, Stanek et al. 2011). However, MC1568 exposure caused P53 hyperacetylation in cell lines when they were co-exposed to etoposide, suggesting that inhibition of class II HDACs may potentiate the stress response induced by another compound (Sen, Kumari et al. 2013).

Altogether these studies suggest that HDAC classes have different roles during development; class I and III HDAC inhibitors are likely to be developmental toxicants. However, additional analysis of limb mesenchyme specific conditional knockout mice or the effects of siRNAs for members of these classes would further identify the exact players involved in mediating these effects.

5.7 Additional future directions

5.7.1 HAT inhibition

The studies presented in this thesis suggest a role for protein acetylation in regulating intracellular signaling and mediating teratogenesis. HDACs, SIRTs and HATs share molecular targets. Although many studies have investigated the developmental toxicity of HDACi, little is known of the effects of HAT inhibition during development and if HAT inhibitors could protect against HDACi-induced malformations. Several HAT inhibitors (HATi) have been characterized (Reviewed in (Furdas, Kannan et al. 2012)); many of

these compounds are not available or have poor bioavailability in vivo. Several commercially available compounds, such as anacardic acid and curcumin, have been shown to have many off-target effects and are considered to be non-specific HATi (Yu, Shen et al. 2008, Tan, Chen et al. 2012). Alternatively, in vivo siRNA or CRISP technology could be used to silence or downregulate HAT specifically. These studies would provide further insights into the role of acetylation protein modification in development and the mechanism of embryonic response to assault.

5.7.2 The role of oxidative stress in VPA-induced skeletal defects

Oxidative stress was shown to play an important role in VPA-induced neural tube defects as these were prevented by exogenous catalase treatment (Tung and Winn 2011). Moreover, in vivo exposure to hydroxyurea, an inducer of oxidative stress and DNA damage, leads to a plethora of skeletal defects (Yan and Hales 2006). Co-exposure of VPA with various anti-oxidants and free-radical spin trap agents, such as N-tertiary-butyl nitrone (PBN), would elucidate the role of oxidative stress in mediating the different effects on chondrogenesis and osteogenesis we observed in this study.

5.7.3 The effects of VPA on P63

P63 belongs to the *p53* family of transcription factors. Mice carrying mutations in the *p63* gene exhibit skeletal malformations and altered COL2a1 expression, suggesting that it plays a role in chondrogenesis (Lu, Abbassi et al. 2013). Retinoic acid exposure reduces p63 in mesenchymal cells; this effect is correlated with a decrease in *Sox9* and *Col2a1* mRNA expression as well as an inhibition of cartilage nodule formation (Wang, Xie et al. 2014). Moreover, p63 is acetylated by PCAF and forms a repressive complex

with HDAC1 and HDAC2 (Ramsey, He et al. 2011, Chae, Kim et al. 2012). Altogether, suggesting p63 might play a role in teratogen-mediated inhibition of chondrogenesis.

5.8 The importance of VPA research and bone development

Although alternatives are available, VPA is still used in certain cases during pregnancy. Moreover, due to its efficacy in the treatment of several types of epilepsy and its tolerable safety profile, VPA is widely used in young and pre-pubertal children (Blanco-Serrano, Otero et al. 1999). Moreover, several HDACi, including VPA, are currently being evaluated as potential cancer therapeutics for childhood and adolescence cancer (Masetti, Serravalle et al. 2011). Skeletal development begins in utero when the skeleton is formed and continues throughout childhood and adolescence; postnatal bone elongation occurs at the epiphyseal cartilage plate comprised of chondrocytes (Emons, Chagin et al. 2011, Mughal and Khadilkar 2011). The effects of VPA on the molecular pathways regulating bone growth at that age remain to be investigated. However, epidemiological studies have shown that exposure to VPA for one year between the ages of 4 and 18 results in a significant decrease in growth (Lee, Wang et al. 2013).

5.9 Conclusions

In conclusion, the studies in this thesis identified early changes in gene expression and intracellular signaling coinciding with changes in activity of histones deacetylases in the developing embryonic limb following in vitro exposure to VPA. Overall, three potential pathways of VPA-induced teratogenesis were identified and shown to be modulated by

acetylation and HDAC inhibition; SOX9, RUNX2 and P53. Many HDACi, including VPA, are currently in clinical trials and will potentially be used as pharmacological anticancer agents in future years. Hence, research into the mechanisms underlying their teratogenic effects remains crucial in the development of prevention strategies and better therapies.

Original Contributions

1. Developing limbs exposed to VPA exhibit reduction in *Sox9* expression and signaling leading to a decrease in expression of the extracellular matrix protein COL2A1. This results in smaller or absent mesenchymal condensations and cartilage template defects leading to limb abnormalities such as oligodactyly, missing phalanges and short metacarpals. This is the first evidence that *Sox9* signaling is targeted by VPA.

2. Upon VPA exposure, *Runx2* signaling is inhibited, leading to a reduction in *Col10a1* mRNA and protein levels. COL10A1 is a major contributor to the extracellular matrix surrounding the hypertrophic chondrocytes that play a major role in limb elongation. Exposure to VPA causes reduction of the digits, ulna and radius. Together, these studies indicate that limb elongation is impaired by VPA.

3. Exposure to VPA causes p53 hyperacetylation and activation in the developing limb. A subsequent increase in cellular apoptosis, consistent with the activation of the intrinsic pathway, was induced by VPA. Cell multiplication and differentiation are pivotal for proper development. Premature or excess cellular death can contribute to the detriment of the limb

4. The effects caused by VPA exposure are correlated with a concentration dependent increase in histone acetylation and histone deacetylase inhibition. Developing limbs exposed to MS275, an HDAC class I inhibitor, exhibited reduction in COL2A1, COL10A1 and COL1A1 and increased apoptosis,

providing further evidence of a correlation between inhibition of specific HDACs and these effects

5. Exposure to MC1568, an inhibitor of HDAC class II, induces minimal teratogenesis and cellular toxicity in the developing limb.

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