Title
The Embryotoxic Effects of Harm Reduction Tobacco Products on Osteoblasts Developing from Human Embryonic Stem Cells

Permalink
https://escholarship.org/uc/item/32z2s123

Author
Sparks, Nicole Renee

Publication Date
2018

License
CC BY 4.0

Peer reviewed|Thesis/dissertation
The Embryotoxic Effects of Harm Reduction Tobacco Products on Osteoblasts
Developing from Human Embryonic Stem Cells

A Dissertation submitted in partial satisfaction
of the requirements for the degree of
Doctor of Philosophy
in
Environmental Toxicology
by
Nicole Renee Sparks

June 2018

Dissertation Committee:
Dr. Nicole zur Nieden, Chairperson
Dr. David Eastmond
Dr. Yinsheng Wang
The Dissertation of Nicole Renee Sparks is approved:


Committee Chairperson

University of California, Riverside
ACKNOWLEDGEMENTS

I am beyond appreciative to have many people in my life that have made this dissertation possible.

I am grateful for the advice and the guidance I received from my principal investigator Nicole I. zur Nieden and the many cups of coffee we enjoyed together. Her knowledge and dedication to stem cell research and toxicology has been inspiring. She has been instrumental in my doctoral career and beyond.

For some of the work presented herein I had technical assistance from Lauren M. Walker (mouse work), Ivann Martinez, Joseph Madrid (in vivo injections), Riley Bottom, and Cristina Soto. Thank you, Lauren, for lending your ear and pastries. Thank you to all my lab mates from the zur Nieden lab and friends made in the Riccomagno lab. We are like family and I have enjoyed my time shared with you all.

Thank you to the Environmental Toxicology doctoral program and my dissertation committee members Dr. David Eastmond and Dr. Yinsheng Wang. A special, thank you to Dawn Loyola who has helped me every quarter of my doctoral career.

I would also like to acknowledge my funding sources: The Dean’s fellowship, fellowships from the graduate research mentorship program, a Cornelius Hopper Diversity and pre-doctoral fellowship as well as a doctoral fellowship from the Tobacco Related Disease Research Program and a fellowship from the International Foundation for Ethical Research. Research funding came from the Tobacco Related Disease Research Program, the John Hopkins Center for Alternatives to Animal Testing as well as the National Institutes for Dental and Craniofacial Research.
I would like to acknowledge Dr. Feodor Price (Harvard University), Dr. Derrick Rancourt (University of Calgary), and Dr. Amander Clark (University of California, Los Angeles) for kindly sharing genomic DNA of hESC and hiPSC lines analyzed in Appendix Fig 1.2.2. I would like to thank Dr. Martín García-Castro for kindly donating SOX10 and PAX7 antibodies. I would like to thank Dr. Prue Talbot (University of California, Riverside) for generously providing our lab with the mainstream and sidestream tobacco smoke solutions used for some of my work. I would also like to thank to Dr. Martin Riccomagno (University of California, Riverside) for kindly providing the pBK-Flex shRNA plasmid and Eric Wong for technical assistance.

Finally, I am forever thankful to my husband Jason. His understanding, encouragement, and amazing patience has been my foundation. Without his support I could have not completed my dissertation. My amazing children, Gavin and Austen Sparks, who have shown patience and understanding and have kept me full of joy and laughter.
ABSTRACT OF THE DISSERTATION

The Embryotoxic Effects of Harm Reduction Tobacco Products on Osteoblasts Developing from Human Embryonic Stem Cells

by

Nicole Renee Sparks

Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, June 2018
Dr. Nicole zur Nieden, Chairperson

Cigarette smoke is a mixture of over 7000 toxic components with at least 69 smoke chemicals producing cancers, suggesting that smoking is harmful to smokers. More importantly, the World Health Organization has estimated that 10% of the world’s population is involuntarily exposed to environmental tobacco smoke, a number that does not include developing embryos. However, tobacco exposure during embryonic development is one of the risk factors for developing a congenital birth defect. An understudied, yet important abnormality caused by maternal smoking is the improper development of the embryonic skeleton, which can result in long-term burdens on the affected child and their family, creating a need for investigation that is addressed in this thesis.

Due to the difficulty in quitting smoking while pregnant, women often look to use harm reduction tobacco products (HRTPs) because they are perceived as a safer alternative in comparison to conventional cigarettes. These products include ultra-filtered cigarettes with reduced carcinogen content, and chewing tobacco, which omits exposure to harmful
combustion products. However, there is a lack of investigation to reveal the molecular mechanisms involved in skeletal embryotoxicity induced by conventional and harm-reduction tobacco, the unraveling of which is another objective of this thesis.

In the process of this investigation, an *in vitro* model based on human embryonic stem cells was developed. These cells are the only currently available cell culture system, which mimics the development of the human embryo. As this thesis shows, their differentiation into osteoblasts *in vitro* can be used to study chemical toxicity and the molecular mechanisms thereof.
# TABLE OF CONTENTS

Acknowledgements ........................................................................................................ iv

Abstract ............................................................................................................................... vi

List of figures ....................................................................................................................... ix

List of tables ......................................................................................................................... xii

Chapter 1: Introduction to stem cells ................................................................................. 1

Chapter 2: Low osteogenic yield in human pluripotent stem cells 
associates with differential neural crest promoter methylation ........................................... 14

Chapter 3: Pluripotent stem cells as tools to assess developmental 
toxicity: diversity instead of consolidation ............................................................................. 50

Chapter 4: Differential predictivity of human pluripotent stem cell 
lines in skeletogenic developmental toxicity assays correlates with 
their innate differentiation bias towards neural crest or mesodermal 
osteogenesis ..................................................................................................................... 73

Chapter 5: Tobacco-induced oxidative stress disrupts osteogenic 
differentiation in human embryonic stem cells................................................................. 119

Chapter 6: Harm-reduction tobacco alters bone development through 
tobacco sensitive transcriptional regulators ........................................................................ 155

Conclusion ......................................................................................................................... 191

APPENDIX 1: Supplemental information ........................................................................... 199

APPENDIX 2: List of differentially expressed genes ......................................................... 208
LIST OF FIGURES

Figure 1.1. Human blastocyst ................................................................. 1
Figure 1.2. Pluripotent stem cell differentiation ..................................... 2
Figure 1.3. Stem cell division and differentiation ..................................... 3
Figure 1.4. Adult stem cell .................................................................... 4
Figure 1.5. Generation and application of induced pluripotent stem cells (iPSCs) ......................................................................................... 6
Figure 1.6. Contributions of neural crest, paraxial mesoderm, and lateral plate mesoderm to bone development ................................................................. 7
Figure 2.1. Cytogenetic, pluripotent, and genetic characterization of human induced pluripotent stem cell (hiPSC) lines ................................................................. 24
Figure 2.2. Osteogenic differentiation of human embryonic stem cells ..... 25
Figure 2.3. Intermediate differentiation in H9 cells is through the neural crest lineage ................................................................................................. 27
Figure 2.4. Osteogenic differentiation in RIV9 and RIV4 human induced pluripotent stem cells (hiPSCs) ......................................................................................... 30
Figure 2.5. Passage-specific analysis of bone parameters and gene expression ................................................................................................. 32
Figure 2.6. Osteoblast origin correlates with methylation of the TWIST1 and PAX7 promoters ......................................................................................... 34
Figure 2.7. Toxicity of 5-fluorouracil (5FU) tested with H9 and RIV9 hPSCs .... 36
Figure 3.1. Embryonic stem cell derivation and differentiation ................. 52
Figure 3.2. Embryonic stem cell test (EST) ................................................ 54
Figure 3.3. Human ESC osteogenic induction ............................................. 60
Figure 4.1. Human ESCs are more sensitive in predicting the developmental toxicity of 13cisRA, a known human teratogen, than mouse ESCs ........................................ 85
Figure 4.2. Differentiation and cell viability endpoints measured with RIV9 hiPSCs .......................................................... 87

Figure 4.3. Human H9 ESCs preferentially undergo neural crest osteogenesis ................................................................. 89

Figure 4.4. Concentration-response curves for representative neural crest-associated inhibitory compounds ................................................................. 91

Figure 4.5. Half-maximal inhibitory doses for cytotoxicity (IC50) as determined from concentration-response curves with MTT assay and differentiation (ID50) with Arsenazo calcium assay and quantitative PCR for TWIST1 ........................................ 93

Figure 5.1. The embryotoxicity of tobacco exposure .......................................................... 124

Figure 5.2. Tobacco dysregulates the expression of osteogenic genes .................. 135

Figure 5.3. Camel sidestream smoke induces apoptosis ........................................ 136

Figure 5.4. Camel sidestream smoke exacerbates apoptosis ................................ 139

Figure 5.5. Tobacco exposure decreases antioxidant activity ............................ 140

Figure 5.6. Tobacco increases production of ROS in differentiating human embryonic stem cells .......................................................... 141

Figure 5.7. Tobacco exposure decreases antioxidant activity ............................ 143

Figure 6.1. pBK-Flex-shRNA plasmid map .......................................................... 162

Figure 6.2. FOXO facilitates osteogenic differentiation ........................................ 167

Figure 6.3. Harm reduction tobacco products diminish the expression of FOXO .......................................................... 169

Figure 6.4. HRTP-mediated nuclear exclusion of FOXO ................................. 171

Figure 6.5. FOXO shRNA clones reduce FOXO protein levels ...................... 172

Figure 6.6. FOXO is essential for osteogenic differentiation .......................... 173

Figure 6.7. Differential expression of genes treated with Camel Blue and Camel Snus tobacco extract .......................................................... 174
Figure 6.8. Highly differentially expression genes sensitive to Camel Blue exposure during osteogenic differentiation .......................................................... 176

Figure 6.9. STE-responsive differentially expressed genes ....................................... 177

Figure 6.10. Genes downregulated unique to Camel Snus tobacco extract treatment .................................................................................................................. 178

Figure 6.11. Camel Blue and Camel Snus tobacco extract regulate different biological processes in differentiating cells ................................................................. 180

Figure 6.12. Camel Blue and Camel Snus tobacco extract negatively affect bone development through separate biological processes .............................................. 181

Figure 7.1. Harm-reduction tobacco products transcriptional regulation during osteogenesis ........................................................................................................... 197

Appendix Figure 1.2.1. Pilot differentiation studies for protocol development ................................................................................................................................. 199

Appendix Figure 1.2.2. Kinetic and passage-specific analysis of additional gene expression associated with osteogenesis and early lineage determination .............. 200

Appendix Figure 1.2.3. Inter-and intra-line variability between different passages of hPSCs used for osteogenic differentiation ................................................................. 201

Appendix Figure 1.2.4. Early lineage determining genes are differentially expressed in the three hPSC lines ............................................................................................... 202

Appendix Figure 1.2.5. Correlation between PAX7 and TWIST1 methylation status and osteogenic differentiation yield across hPSC lines ......................................................... 203

Appendix Figure 1.4.1. Testing of three control chemicals in the hESC-ESTo ........ 205

Appendix Figure 1.4.2. The effects of 13cisRA on somatic cells ............................. 206

Appendix Figure 1.4.3. TWIST1 mRNA expression as endpoint in the hESC-ESTo ................................................................................................................................. 207
LIST OF TABLES

Table 4.1. Half-maximal inhibitory concentrations for all tested endpoints and prediction of embryotoxicity classes ................................................................. 94

Table 5.1. Apoptosis-related genes examined with the RT² Profiler™ qPCR array ..................................................................................................................... 132

Table 5.2. Primer sequences used in this study. Ta, annealing temperature ............................................................................................................................... 134

Table 6.1. Oligonucleotide sequences ............................................................................................................................ 164

Appendix Table 1.2.1. Oligonucleotide sequences used in this study. Ta, annealing temperature .............................................................. 204
CHAPTER 1

Introduction to stem cells

Stem cells are unique cells, regardless of their source of origin, that are defined by the ability to self-renew and unspecialized with the capacity to differentiate into different cell lineages. The self-renewal and differentiation properties of stem cells are what make them different from the majority of somatic cells that are committed to a specific function, such as a calcifying osteoblast and a beating cardiomyocyte. As such, stem cells are a powerful developmental tool and can aid in the understanding of tissue development, cell-based therapies, drug discovery, screening of toxicants, and understanding of diseases and birth defects (Tandon and Jyoti, 2012).

One such stem cell type, the embryonic stem cell (ESC), is derived from the inner cell mass (ICM) of a pre-implantation blastocyst, a 3 to 5-day old embryo. The blastocyst is formed early in development after the first lineage commitment. Here, a subset of cells specifies towards the trophectoderm, the extraembryonic tissue like the placenta, and the remaining cells becoming the ICM, which develops into the future embryo (Fig 1.1). ESCs do not have the ability to differentiate into the extraembryonic tissue, but instead are

Figure 1.1. Human Blastocyst. Blastocyst showing the inner cell mass (ICM) and trophectoderm. Zhang et al., 2009.
Figure 1.2. Pluripotent stem cell differentiation. Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst. ESCs have the ability to be propagated in culture and can differentiate to all cell types from the three germ layers. https://ltsa.sheridancollege.ca/apps/human-anatomy/quiz_labeling_femur.html?bgImage=; https://www.sciencelearn.org.nz/resources/1917-muscle-structure-muscle-under-the-microscope; http://jonlieffmd.com/blog/how-many-different-kinds-of-neurons-are-there; http://trollpasta.wikia.com/wiki/File:Red-blood-cells-on-white.jpg; https://noticieros.televisa.com/ultimas-noticias/cientificos-unam-crean-metodo-mas-preciso-diagnosticar-higado-graso/
pluripotent: they can give rise to cell types from all three germ layers (i.e., ectoderm, mesoderm, and endoderm) (Fig 1.2; Itskovitz-Eldor et al., 2000) and can undergo extensive self-renewal and proliferation in vitro. Due to these properties, ESCs can be utilized for prenatal and postnatal studies in the pluripotent and differentiated states, respectively.

In 1981 came the first reports of the derivation and methods of growing mouse ESCs (mESCs) (Martin, 1981; Evans and Kaufman, 1981). Early mESC studies provided a foundational understanding of embryonic stem cell biology and advanced appropriate culture techniques to facilitate eventual derivation of human ESCs (hESCs) from cryopreserved human blastocysts (Thomson et al., 1998). Prolonged pluripotency and proliferation of ESCs is attributed to the expression of stage-specific pluripotency markers, high telomerase activity, and the absence of lineage markers. Human ESCs express *POU5F1*, *NANOG*, *SOX2*, *SSEA-3*, and *SSEA-4* for pluripotency and downregulate the respective genes to initiate differentiation (Hadjimichael et al., 2015). The unlimited self-renewal of hESCs is associated with maintenance of long telomeres, which somatic cells

![Figure 1.3. Stem cell division and differentiation. Through asymmetrical division, the stem cell can self-renew maintaining stemness or choose to differentiate and lose stemness. During differentiation the cell will go through progenitor states before becoming a somatic cell (terminal differentiation).](image-url)
do not possess (Huang et al., 2011). In somatic cells, telomere shortening eventually leads to cellular senescence and apoptosis.

Mouse and human ESCs can remain undifferentiated and self-renew under the correct culture condition. When ESC colonies are able to touch or clump together, this can lead to spontaneous differentiation. Their differentiation can be directed into specific cell types (i.e., osteoblasts, cardiomyocytes, chondrocytes and neurons) by changing their culture medium to suppress pluripotency and upregulate cell specific genes (zur Nieden et al., 2003; zur Nieden et al., 2005; Okabe et al., 1996). When ESCs progress towards a particular lineage, they undergo an asymmetrical division process that creates a progenitor cell. Progenitor cells are distinct from ESCs in that they possess limited proliferation capacity and reduced potency. Eventually, these cells will terminally differentiate into a mature somatic cell (Fig 1.3).

In contrast to pluripotent stem cells, adult stem cells (ASCs) are unspecialized cells derived from specific somatic tissues (Fig 1.4). ASCs have been identified in many tissues from all germ layers and can self-renew and differentiate into cell types they reside in, having multipotent or unipotent potential. For instance, a hematopoietic stem cell (HSC) resides in the bone marrow. These cells can only form blood cells. Thus, the role of ASCs
in the adult body is to maintain homeostasis and repair damaged tissue. ASCs possess limited self-renewal capacity and prefer to remain quiescent until needed. Though restricted into what cells types they can become, ASCs do have clinical relevance. In particular, HSCs and mesenchymal stem cells (MSCs) have been used for blood and bone regeneration (Reya et al., 2001). Due to their harvest not destroying an embryo, ASCs do not have the same ethical concerns as ESCs.

It has been the ethical concerns associated with the use of ESCs that has prohibited some countries from allowing ESC research (Dhar and Hsi-En, 2009) and alternative ways to create ethically unchallenged pluripotent cells have been sought. In a technique called somatic cell nuclear transfer (SCNT), a somatic cell nucleus is transferred into an enucleated oocyte, activated to proliferate and ESC-like cells derived from the emerging blastocyst (Wilmut et al., 1997; Gurdon and Uehlinger, 1966). SCNT offered the potential to generate various cells types for research and therapeutic usage, however proved to be labor intensive, inefficient, and raised concerns of human cloning. This changed with the generation of induced pluripotent stem cells (iPSCs) (Takahashi et al., 2006). Induced PSCs are generated through the viral transduction of mouse somatic cells, often fibroblasts, encoding for four transcription factors (Pou5f1, Sox2, Klf4, and c-Myc; Takahashi et al., 2006). Using the same technique, human induced pluripotent stem cells (hiPSCs) were generated a year later (Takahashi et al., 2007). Similar to hESCs, hiPSCs can self-renew and can differentiate into cell types from all three germ layers. Human iPSCs are hypothesized to be a powerful tool to study development and patient-specific diseases that
can be globally adapted (Fig 1.5). However, evidence has shown differences in methylation patterns and iPSCs retaining signatures of their tissue of origin (Lister et al., 2011) as also found in this thesis (chapter 2).

Osteogenesis from ESCs

Osteogenesis is the complex process of bone formation that occurs during embryonic development, bone homeostasis in adults, and bone remodeling after an injury. During embryonic development, bone develops in two ways: intramembranous ossification and endochondral ossification. Neural crest derived osteogenic precursor cells undergo intramembranous ossification to give rise to the flat bones (parts of the skull and pelvis). The long bones, like the axial and appendicular skeleton, are derived from a mesoderm
origin that often, but not exclusively, uses endochondral ossification (Fig 1.6, reviewed in Keller and zur Nieden, 2011). Endochondral ossification has a cartilaginous precursor framework that is eventually replaced with bone (Provot et al., 2013; Gilbert, 2000). The formation and maintenance of bone is tightly regulated between osteoblasts, the cells that synthesize a calcified and mineralized extracellular matrix (ECM), and osteoclasts, the bone absorbing cells. Regardless of mesoderm or neural crest developmental origin, bone progenitor cells condense at locations of skeletal formation. These future sites of bone formation are packed with MSCs which will differentiate into the osteoblasts and later the terminally mature osteoblasts, known as osteocytes. Osteoblast differentiation is controlled by stage (proliferation, differentiation, mineralization, and maturation) specific expression of regulatory proteins.

Factors that control osteoblastogenesis from ESCs include ascorbic acid, β-glycerophosphate and 1,25-(OH)₂ vitamin D₃ (zur Nieden et al., 2003). Pioneering studies
from our laboratory showed that during mESC osteogenesis osteogenic genes are regulated in a temporal manner equivalent to embryonic and MSC osteogenesis: alkaline phosphatase (ALPL) expression is followed by CBFA1, an osteogenic master regulatory transcription factor (Ducy et al., 1997) coding for runt-related transcription factor 2 (RUNX2), as well as osteopontin (OPN). Later during the differentiation, bone sialoprotein (BSP) and osteocalcin (OCN) are expressed marking mature osteoblasts, then followed by CAPG and DESTRIN, which are associated with osteocytes (Ehnes et al., 2015). Mis-regulation at any step during osteogenesis can lead to a wide array of skeletal disorders and diseases (zur Nieden et al., 2005, 2007).
Aims and Objectives

Epidemiological studies suggest cigarette smoking as a probable environmental factor for a variety of congenital anomalies (Surgeon General, 2010), including low bone mass (Godfrey et al., 2001) and increased fracture risk (Parviainen et al., 2017), both of which affect skeletal health. However, there is a lack of investigation to reveal the molecular mechanisms involved in tobacco-induced skeletal toxicity. This thesis aims to provide knowledge on how tobacco exposure can perturb osteoblast differentiation by addressing four specific aims:

Specific Aim 1: Develop a protocol to differentiate human ESCs and iPSCs into osteoblasts \textit{in vitro} (addressed in chapter 2)

Specific Aim 2: Develop an \textit{in vitro} developmental osteotoxicity screening model using hESCs and hiPSCs to predict and identify developmentally toxic compounds (addressed in chapters 3 and 4)

Specific Aim 3: Determine the impact of tobacco-induced oxidative stress and downstream transcriptional changes during osteogenic differentiation (addressed in chapters 5 and 6)
References


CHAPTER 2

Low Osteogenic Yield in Human Pluripotent Stem Cells Associates with Differential Neural Crest Promoter Methylation

Nicole Renee Lee Sparks, Ivann Kenneth Carvajal Martinez, Cristina Helen Soto, Nicole Isolde zur Nieden

Abstract

Human pluripotent stem cell-derived osteoblasts possess great potential for utilization in bone disorder elucidation and repair; yet, while the general ability of human pluripotent stem cells to differentiate into osteoblasts and lay down bone-specific matrix has been shown, previous studies lack the complete characterization of the process whereby such osteoblasts are derived as well as a comparison between the osteogenic efficiency of multiple cell lines. Here, we compared the osteogenic potential of two human induced pluripotent stem cell lines (RIV9 and RIV4) to human H9 embryonic stem cells. Generally capable of osteogenic differentiation, the overall osteogenic yield was lower in the RIV9 and RIV4 lines and correlated with differential expression of osteocalcin (OCN) in mature cultures and PAX7 and TWIST1 during early differentiation. In the undifferentiated cells the promoters of the latter two genes were differentially methylated potentially explaining the variation in differentiation efficiency. Furthermore, the expression signatures of selected neural crest and mesodermal genes and proteins suggested that H9 cells
preferentially gave rise to neural crest-derived osteoblasts, whereas the osteoblasts in the RIV9 cultures were generated both through a mesodermal and a neural crest route albeit each at a lower rate. These data suggest that epigenetic dissimilarities between multiple PSC lines may lead to differences in lineage derivation and mineralization. Since osteoblast progenitors from one origin inadequately repair a defect in the other, these data underscore the importance of screening human pluripotent stem cells lines for the identity of the osteoprogenitors they lay down.

Introduction

The \textit{in vitro} differentiation of human embryonic stem cells (hESCs) offers an invaluable source to study cellular development, the etiology of degenerative diseases, and the regeneration of dysfunctional tissues [1-4]. Human ESCs are exceptionally appropriate for the \textit{in vitro} generation of specialized cells due to their pluripotency. Researchers have developed \textit{in vitro} protocols that differentiate ESCs from mouse [5-7] and human [79] into functional osteoblasts that can mineralize their extracellular matrix (ECM), a hallmark of bone formation. Directed differentiation of ESCs towards an osteoblast lineage has been achieved using dexamethasone (DEX), which triggers the expression of the osteogenic genes runt-related transcription factor 2 (\textit{RUNX2}) and osteocalcin (\textit{OCN}) [10, 11]. Our group routinely uses the active exogenous factor 1α,25 dihydroxy vitamin D\textsubscript{3} (VD\textsubscript{3}) to enhance commitment of mouse or primate ESCs towards the osteoblast lineage [6, 7, 12]. However, the ethical concerns surrounding the use of hESCs have held back exploring the great expectations otherwise associated with hESCs. Human induced
pluripotent stem cells (hiPSCs), generated from the reprogramming of somatic cells, are also truly pluripotent cells and provide an alternative mean to obtain ES-like cells [13,14]. While believed to be broadly equivalent to hESCs based on morphology and gene expression [15-17], several reports have identified hiPSC lines to be different from hESCs in DNA methylation and gene expression signatures [18-21].

Increasingly, the capacity of iPSCs to differentiate into osteoblasts is at the center of studies, as these cells may be used in cell-based replacement therapies or embryotoxicity screening assays [22, 23]. To direct osteogenic differentiation in hiPSCs prior studies employed DEX addition to the culture medium or seeding these cells on three-dimensional (3D) scaffolds [24-32]. While these studies have provided evidence that hiPSCs are generally capable of generating osteoblasts they have not characterized whether they do so with a similar efficiency as hESCs and whether they do so through the same process. This is important, because osteoblasts may originate from the neural crest or the mesoderm, the former having superior grafting abilities than the latter [33, 34]. Similarly, a culture process which derives osteoblasts from the neural crest may not be suitable to uncover embryotoxicants that cause limb malformations in vivo.

As it is thus clearly necessary to elucidate the efficacy of hiPSC technology prior to routine experimental utilization of these cells [35], here we compared the osteogenic differentiation potential of two hiPSC lines, RIV9 and RIV4, with hESCs of the H9 line. Although RIV9 and RIV4 cells were generally capable of differentiating into functional matrix-calcifying osteoblasts when induced with VD₃, both of the hiPSC lines tested exhibited a lower potential to yield osteoblasts than the H9 cells as determined by bone-
specific mRNA expression and quantification of calcium accumulation. Despite their lower differentiation potential, osteogenically induced RIV9 cells were equally sensitive to the strong embryotoxicant 5-Fluorouracil indicating their potential usefulness for the screening and identification of putative skeletal toxicants.

However, in H9 hESCs, VD₃ induction seemed to cause osteoblasts to form from a source of neural crest cells, while RIV9 osteoblasts seemed to be specified primarily from mesoderm and to a lesser degree the neural crest, whereas RIV4 cells seemed to prefer a neuroepithelial path. Differentiation efficiency and route was not inherently associated with the type of pluripotent stem cell, but rather seemingly correlated with the methylation state of neural crest promoters that was found before differentiation was initiated. In summary, our results indicate that before individual hPSC lines can come to use in orthopedic regenerative medicine or for the study of molecular mechanisms accompanying osteogenic differentiation, they need to be thoroughly characterized for the lineage origin they derive osteoblasts from.

Materials and Methods

*Human pluripotent stem cell lines*

Cells of the hESC H9 line were acquired from WiCell (WiCell Research Institute). RIV4 and RIV9 hiPSCs were generated from foreskin fibroblasts (HFF-1, ATCC) by retroviral integration of *OCT4, KLF4* and *SOX2* at the University of California Riverside’s Stem Cell Core Facility [36, 37]. To disrupt expression of T/Brachyury, human pluripotent stem cells (4 x 10⁵ cells) were transfected with 0.3 µg brachyury CRISPR/Cas9 KO plasmid
(h; sc-416539) and 0.3 µg brachyury HDR plasmid (h; sc416539-HDR) from Santa Cruz using Effectene Transfection Reagent (Qiagen 301427). After 72 hours, transfectants were selected with 150 µg/ml puromycin (Sigma-Aldrich P8833) for 3-5 days. Resistant colonies that were double positive for green and red fluorescent protein were picked and expanded for analysis. Osteogenic differentiation was conducted from those clones, in which a reduction in BRACHYURY protein expression during differentiation was confirmed. Control clones were generated using 0.3 µg Control CRISPR/Cas 9 plasmid. All cells were maintained on Matrigel (BD Biosciences) treated dishes in mTeSR® (Stem Cell Technologies) in 5% CO₂ and at 37°C. Pluripotent colonies were passaged every 5 days upon reaching 70% confluency by dissociating cells with accutase and a cell scraper.

**Karyotyping**

Standard G-bandning type chromosome and cytogenetic analysis were carried out at the WiCell Research Institute.

**Osteogenic Differentiation of hPSCs**

Confluent pluripotent colonies (designated day 0) were switched to control differentiation medium composed of Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) 15% FBS (Atlanta), 1% non-essential amino acids (NEAA; Gibco), 1:200 penicillin/streptomycin (Gibco), and 0.1 mM β-mercaptopethanol (Sigma). On day 5, control medium was supplemented with 10 mM β-glycerophosphate (Sigma), 50 µg/ml ascorbic acid (Sigma), and 50 nM 1,25(OH)₂ Vitamin D₃ (Calbiochem) [38].
**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde for 30 min at 4°C. After washing and permeabilizing (intracellular markers only) with 0.1% Triton-X 100 (Sigma) for 15 min, cells were incubated in primary antibody diluted in PBS/10% FBS overnight at 4°C. Primary antibodies used were POU5F1 (EMD Millipore, ab3209), NANOG (Cell Signaling 4903), SOX2 (Cell Signaling, 3579), SSEA4 (EMD Millipore, MAB4304), OCN (Abcam, ab1857), p75NTR (Abcam, ab8874), SOX10 (Santa Cruz, SC17342), TWIST1 (Abcam, ab50887), PDGRFα (Abcam, ab61216), and PAX7 [39] (mIgG1, Developmental Studies Hybridoma Bank). Cells were then washed and incubated in the appropriate secondary antibody (AlexaFluor conjugated) for 2h. Cells were observed on a Nikon fluorescent microscope.

**Histochemical analysis**

Cultures were washed with PBS and fixed in 4% paraformaldehyde at 4°C for 30 min. For von Kossa stain, fixed samples were overlaid with silver nitrate solution (Ricca Chemical Company) and illuminated under an intense light source for 1 h. Cultures were fixed with 5% sodium thiosulfate (Red Bird Service) for 2 minutes. Cells were also washed with PBS and overlaid with 2% w/v Alizarin Red S solution (Sigma-Aldrich) for 5 minutes to visualize calcium-rich areas. Prior to imaging, cultures were washed 3X with water followed by ascending alcohol washes (70%, 80%, 90%, 100% ethanol).
Characterization of bone: Alkaline phosphatase (ALP), inorganic phosphate, and calcium assays

Cells were harvested in radioimmunoprecipitation (RIPA) buffer. Activity of the ALP enzyme and calcium deposition were determined and calculated as previously described [40]. Free inorganic phosphate ($P_i$) was measured with the PiPer phosphate assay (Molecular Probes, Invitrogen) per the protocol of the manufacturer and as used by Majumdar et al. [41]. The total free inorganic phosphate in the sample was taken from a potassium phosphate standard curve. All measurements were normalized to the total protein content of the sample measured with the Lowry method.

RNA isolation and quantitative PCR

Total RNA was extracted with the NucleoSpin RNA II protocol (Macherey Nagel). RNA concentration was determined with a NanoDrop® 1000 spectrophotometer (Thermo Scientific) at 260 nm. 25 ng of total RNA was used for cDNA synthesis with a mastermix including 1X reaction buffer, 0.5 mM dNTPs, 20 U/µL RNase inhibitor, 0.8 U/µL reverse transcriptase, and 1 µM random primer. 25ng cDNA transcripts were used for quantitative polymerase chain reaction (qPCR) with SYBR green on the MyiQ cycler (BIO-RAD). Reactions were setup for 10 minutes of denaturing at 94°C, followed by 40 cycles of denaturing at 94°C, and annealing/elongation at 60°C for 45 seconds. The n-fold expression in target samples was calculated with the $\Delta\Delta C_T$ method by standardizing $C_T$ values to GAPDH expression [42]. Primer sequences used in this study can be found in Appendix Table 1.2.1. Pre-designed primer pairs for GOOSECOID (GSC;
Hs00906630_g1), PAX6 (Hs00240871_m1), and SOX17 (Hs00751752_s1) were purchased from Life Technologies. All other primers were from Leung et al., 2016 [43].

**Methylation-specific (MS) PCR Analysis**

To analyze the methylation status of the TWIST1 and PAX7 promoters, cells were collected in lysis buffer (100 mM NaCl, 0.5% SDS, 20 mM Tris pH7.5, 50 mM EDTA) with 0.2 mg of proteinase K (Sigma) and incubated at 60°C overnight. DNA was isolated by standard phenol-chloroform and isopropanol precipitation methods and quantified with a NanoDrop® 1000 spectrophotometer. Bisulfite conversion was performed from 200 ng of genomic DNA with the EZ DNA Methylation-Lightning Kit (Zymo). The methylation status of the PAX7 and TWIST1 promoters was then determined from the eluted DNA using the MS-nested PCR method [44]. Primer sequences for first and second rounds of amplification for both methylated and unmethylated DNA are listed in Appendix Table 1.2.1 and are as previously published [44]. Cycling conditions for PAX7 were 10 minutes at 95°C, followed by 40 cycles at 94°C for 25 seconds, 60°C for 25 seconds, and 72°C for 20 seconds. A final elongation of 2 minutes at 72°C followed. The first and second reactions for TWIST1 were setup for 10 minutes at 95°C, followed by 40 cycles for round one and 32 cycles in round 2 at 94°C for 25 seconds, 63.9°C for 25 seconds, 72°C for 20 seconds, and a final 2 minute elongation at 72°C. Amplified products were observed in a 2% agarose gel using ethidium bromide for detection.
**MTT assay**

Cell viability was determined with 3-[4,5dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide (MTT) as described previously [45] and absorbance was read at 595 nm in an iMark™ microplate reader (BIORAD).

**Video bioinformatics**

Phase contrast microscopic images of differentiating hPSCs were automatically acquired at a magnification of 4X every 12h utilizing the BioStation CT (Nikon Corporation, Tokyo, Japan). For each 24-well dish well ten reference points of interest were randomly selected as representative view fields. Raw images were processed, enhanced and segmented using MATLAB software generated recipes (Mathworks, MA, U.S.A) using a manual threshold of 33. Pixels below the threshold were removed to create segmented images of calcified regions. Remaining pixels were counted to quantitatively represent the degree of calcification from each image. The calcification rate was determined from the number of calcified pixels in each image at t<sub>x</sub> subtracted from the number of calcified pixels at t<sub>0</sub> and divided by the hours of elapsed time.

**Toxicity Testing**

5-Fluorouracil (5FU) was obtained from Sigma-Aldrich and a stock solution prepared in dimethyl sulfoxide. Differentiation of hPSCs was initiated as described above and cells were treated for 20 days of osteogenesis with five concentrations of 5FU and a solvent only control. 5FU was replenished with each media change.
**Statistical Analysis**

All experiments were performed from 3 independent passages, each consisting of either three or five biological replicates (different wells). Video bioinformatics assessment was performed from a total of 30 areas within three independent culture wells per replicate. For single and multiple comparisons, an unpaired student’s T-test and a One-Way ANOVA with Holm-Sidak post-hoc analysis was performed, respectively. A p-value of 0.05 or smaller was considered statistically significant (SigmaPlot, Systat Software, San Jose, CA).

**Results**

**Pluripotency assessment of cell lines used**

To apply hiPSC technology in the cell-based replacement of diseased or injured bone tissue or the screening for skeletal toxicity and teratogenicity, the osteogenic differentiation potential of human induced pluripotent stem cells was first determined. To rule out that eventual differences in osteogenic differentiation capacity stemmed from differences in their pluripotency status, H9 hESCs and RIV4 and RIV9 hiPSCs were first compared for expression of pluripotency associated markers and absence of those associated with germ layer differentiation.

All lines displayed similar morphology and both hiPSC lines showed a normal karyotype (Fig. 2.1A, B). In the undifferentiated state, RIV9 and RIV4 lines expressed POU5F1, SOX2, and NANOG transcription factors, which localized to the nucleus, and the membrane associated stage-specific embryonic antigen 4 (SSEA4; Fig. 2.1C). A quantitative comparison of pluripotency marker mRNA levels showed no statistical
difference between the RIV and the H9 lines (Fig. 2.1D). Upon withdrawal of pluripotency conditions, all lines up-regulated the expression of tri-lineage genes (Fig. 2.1E-G), which were found at very low levels in the undifferentiated cells, concomitant with a reduction in pluripotency mRNAs with increased time in differentiation conditions (DMEM+FBS). These data indicated that all lines used in this study showed general tri-lineage potential facilitating the assessment of their osteogenic differentiation potential.

Figure 2.1. Cytogenetic, pluripotent, and genetic characterization of human induced pluripotent stem cell (hiPSC) lines. (A): Morphology of H9 human embryonic stem cells (hESCs) and RIV9 and RIV4 hiPSCs. (B): Cytogenetic G-band analysis for RIV9 and RIV4 hiPSCs (46, XY). (C): Immunofluorescence staining for pluripotent markers. Insets: Nuclear counterstaining with 4′,6-diamidino-2-phenylindole. (D): Quantitative polymerase chain reaction (qPCR) for mRNA expression of pluripotency and lineage markers on undifferentiated hPSCs. Expression is normalized to H9 hESCs and standardized to GAPDH, n = 3 independent samples ± SD. (E-G): qPCR mRNA expression of the same set of mRNAs on differentiating hPSCs, normalized to GAPDH, n = 3 independent samples ± SD. *p < 0.05, Student's t test compared with expression in undifferentiated cells (mTeSR). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; T/BRA, T-brachyury.
Characterization of osteoblast cells derived from hESCs

For osteogenic induction, we adopted a monolayer overgrowth culture approach previously described by Karp et al. [10] (for schematic see Fig. 2.2A) after attempts to execute an embryoid body-based protocol failed due to excessive cell death after replating (Appendix Fig. 1.2.1A). We selected VD3 as an osteogenic supplement as it led to high levels of alkaline phosphatase (ALP) enzyme activity and superior calcification over DEX induction in pilot experiments (Appendix Fig. 1.2.1B).

Upon VD3 induction, differentiating ESCs showed black appearing deposits, which represent the calcification of the extracellular matrix, a hallmark of bone development from stem cells [46] (Fig. 2.2B). Cultures were positive for the major non-collagenous bone protein OCN and positive deposition of calcium ions was depicted by Alizarin Red S and Von Kossa.

![Figure 2.2](image.png)

**Figure 2.2.** Osteogenic differentiation of human embryonic stem cells. (A) Schematic representation of the osteogenic induction protocol. (B) Immunostaining with an osteocalcin antibody reveals presence of bone matrix in the vicinity of black deposits. Alizarin red S staining detected calcium ions in the same areas. Scale bars = 100 µm. (C) Quantitative measurement of deposited calcium over time, n = 5 independent replicates ± SD, *p <0.05 Student's t test compared with d0. (D) Quantitative mRNA analysis of RUNX2, SATB2, and OCN, normalized to GAPDH (n = 3 independent samples ± SD), *p<0.05 one-way analysis of variance over day something missing here. Triplicates were pooled for reverse transcriptase polymerase chain reaction analyses of the osteocyte genes CAPG and DESTRIN (inset). Abbreviations: Alizarin red S, DAPI, 4',6-diamidino-2-phenylindole; ESC, embryonic stem cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OCN, osteocalcin.
stains (Fig. 2.2B). Quantitatively, the levels of deposited calcium rose over non-induced cultures by 10 days of the in vitro differentiation and had reached levels that were 78-fold higher than the non-osteogenic cells by day 30 (Fig. 2.2C). At late stages of osteogenesis, osteoblast-specific expression of SATB2, RUNX2 and OCN transitioned into osteocyte specific expression of CAPG and DESTRIN [48] (Fig. 2.2D).

During early differentiation, the co-expression of SOX10/PAX7 and SOX10/TWIST suggested the presence of neural crest cells [39] (Fig. 2.3A), while these were absent in uninduced control cultures. Further, p75NTR positive cells, often associated with migrating cranial neural crest cells [39, 49], were more abundant than in spontaneously differentiating cultures, and a few days later cells became positive for PDGFRα, which associates with mesenchymal cells, a subsequent step in maturation (Fig. 2.3B). In line with this observation the expression of PAX7 and TFAP2α, two neural plate border specifier genes [48-51], were over 470- and 3160-fold up-regulated over undifferentiated cells (Fig. 2.3C). Mimicking the timely expression during neural crest development, SOX10 and ZIC3 were expressed slightly later. Subsequently, DLX6, which marks osteoprogenitors from the neural crest was significantly up-regulated over undifferentiated cells (Fig. 2.3C). In contrast, T/Brachyury (T/BRA, a pan-mesodermal gene) [52], peaked simultaneously with PAX7 on day 5 of differentiation (Fig. 2.3D), but remained ~140-fold lower than that of PAX7. PAX7 expression was also 98- and 49-fold greater than the levels of the neuroepithelial SOX1 and PAX6, respectively (Fig. 2.3E), suggesting that hESCs primarily differentiated through a neural crest route.
Osteogenic Analysis of hiPSCs

We next tested whether the RIV9 and RIV4 cells also responded to VD₃ with osteogenic differentiation. On day 30 of the culture process, both the RIV9 and RIV4 osteogenic cultures indeed displayed the characteristic black deposits in brightfield microscopy indicative of the mineralized extracellular matrix produced by osteoblasts (Fig.)

Figure 2.3. Intermediate differentiation in H9 cells is through the neural crest lineage. (A, B) Double staining with antibodies against SOX10/PAX7, SOX10/TWIST1, and single stains with p75NTF, and platelet-derived growth factor receptor α at indicated time points of differentiation. Scale bar = 100 µm. (C–E) Quantitative mRNA analysis of early lineage genes, normalized to GAPDH, n = 3 independent samples ± SD. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PDGFR α, platelet-derived growth factor receptor α; T/BRA, T-brachyury.
2.4A). This was further supported by positive immunostaining for OCN, Alizarin Red S, and von Kossa stains. Both RIV lines also showed measurable levels of inorganic phosphate and calcium (Fig. 2.4B, C). While these data suggested that all tested lines were generally capable of differentiating into osteoblasts, we noted an inconsistency in the mineralization process between H9, RIV9 and RIV4 during our studies. For example, inorganic phosphate was significantly decreased in the RIV4 cell line compared to the H9 cells (Fig. 2.4C, P<0.05), while both RIV4 and RIV9 lines showed less calcium content on day 20 (P<0.05) by multiple decades (Fig. 2.4B). Consequently, the Ca/P ratio, an important parameter in the determination of bone quality [53], was lower in both tested hiPSC lines, not even reaching values of 0.58 associated with fetal bone [53] (Fig. 2.4D). Only the Ca/P ratio for the H9 cells was determined at a close to physiological 2.6:1 [53-56].

We next examined when this difference first manifested itself. Morphological assessment suggested that both RIV4 and RIV9 cells calcified less than the H9 cells (Fig. 2.4D), and it appeared as if black deposit was visible earlier than in the H9 cells. Quantitative calcium measurements revealed significantly more calcification in RIV9 hiPSCs than in H9 hESCs on day 5, a pattern that reverted by day 15 (Fig. 2.4E). Instead, the inorganic phosphate content did not show any discernable pattern over a 20-day period (Fig. 2.4E).

To quantitatively determine this apparent difference in kinetics, we applied an innovative time-lapse morphometric assessment taking advantage of the black appearance of the calcium deposits in images [47]. Images of differentiating cultures were acquired
every 12 hours over the course of 20 days and image segmentation applied to separate black pixels from the background. The remaining black pixels were used as a measure for calcification. Both RIV lines exhibited fewer dark pixels confirming a lower degree of calcification in the hiPSC lines (Fig. 2.4F). Calculating the calcification rate by charting the addition of black pixels as a function of elapsed time revealed that RIV4 and RIV9 appeared to calcify earlier than H9 cells (Fig. 2.4G). Together these data imply that hiPSC differentiations varied in the rate that calcification occurred at, with considerably lower osteogenic potential in the RIV4 and RIV9 lines.

Passage-to-passage analysis of osteogenic hPSC cultures

We next aimed to elucidate whether the noted variability in differentiation yield was due to differences in differentiation potential between passages or truly inherent in the specific line tested. To examine this variability more closely, we next compared three experiments inoculated from subsequent passages of cells (denoted by Roman numerals I-III) for osteogenic yield and levels of expression of genes associated with different levels of culture/osteoblast maturation.
Figure 2.4. Osteogenic differentiation in RIV9 and RIV4 human induced pluripotent stem cells (hiPSCs). (A) Photomicrographs of unstained and stained osteogenic cultures from H9 human embryonic stem cells and RIV9 and RIV4 hiPSCs on day 30 of differentiation. Matrix mineralization detected with OCN, Aliz S, and Von Kossa stains. (B) The 20-day-old osteogenic hPSCs deposit significant levels of calcium ions over spontaneously differentiating cultures (n = 5 independent samples ± SD). *p<0.05 one-way analysis of variance (ANOVA) over spontaneous differentiations. (C) Levels of inorganic phosphate levels measured with the PiPer assay on day 20 content, n = 5 independent samples ± SD. #p<0.05 one-way ANOVA over H9. Bottom graph depicts calcium:phosphate ratios determined from Ca2+ and PiPer assays, day 20, n = 3 independent replicates ± SD. *p<0.05 one-way ANOVA versus H9. (D) Morphological time course of hPSC osteogenic differentiation reveals differences in calcification yield between different hPSC lines. (E) Calcification time course analysis shows heterogeneity between osteogenesis in different hPSC lines. Levels of inorganic phosphate also vary depending on differentiation day; n = 3 independent replicates ± SD. #p<0.05 RIV9-H9; *p<0.05 RIV4-H9, one-way ANOVA. (F, G) RIV9 and RIV4 cultures begin their calcification process earlier than H9 cells as determined by video bioinformatics analysis, n = 10 technical replicates from three biological replicates ± SEM. Abbreviations: Aliz S, alizarin red S; DAPI, 4′,6-diamidino-2-phenylindole; OCN, osteocalcin.
The overall yield in calcium deposit followed a similar pattern of continuous up-regulation in all three cell lines but was significantly lower in both RIV lines (H9>RIV9>RIV4, Fig. 2.5A). At the gene expression level, consistent patterns of expression that correlated between the three cell lines and with the calcification data were found for OCN, PAX7 and TWIST1 with the latter inversely correlating to calcification (Fig. 2.5B-D).

In contrast, no other examined genes exhibited any clear time- or cell-line dependent pattern of gene expression, although RUNX2 and OPN indicated continuous maturation over time in the H9 cultures despite one out of the three passages under-expressing when compared to the other two (Appendix Fig. 1.2.2A, B). Similarly, no time-dependent pattern could be observed for ALP enzyme activity or inorganic phosphate content (Appendix Fig. 1.2.2E, F).

To test whether the observed differences between the three cell lines were correlative to passage variability, we next calculated the n-fold differences in gene expression, mineral content and enzyme activity across all time points and graphed them as box plots Appendix Fig. 1.2.3). These indicated that although some of the endpoints showed higher variability in the RIV9 cells, most of the endpoints exhibited equal variations in all cell lines, and none correlated with overall osteogenic yield.
Figure 2.5. Passage-specific analysis of bone parameters and gene expression. (A) Calcium content in hPSC cultures inoculated from three different passages (I–III). Data points represent five technical replicates for each biological passage shown ± SD. *p<0.05 passage II over I; #p<0.05 passage III over I; Δp<0.05 passage III over II. (B–D) The mRNA expression of osteogenic genes was determined with quantitative polymerase chain reaction and normalized to GAPDH, n = 3 ± SD. RIV9 and RIV4 human induced pluripotent stem cells failed to show definitive osteogenic patterns. *p <0.05 passage II over I; #p <0.05 passage III over I; Δp<0.05 passage III over II, one-way analysis of variance. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OCN, osteocalcin.
Calcification ability of hPSCs correlates with PAX7 and TWIST1 expression and methylation status

Based on the association of PAX7 and TWIST1 expression measured in H9 ESCs (Fig. 2.2G) with neural crest lineage determination, and the correlative calcification kinetics found for H9 ESCs, we next comparatively measured the difference in mRNA expression patterns of multiple genes associated with the neural crest as well as mesodermal lineage across the three cell lines. PAX7, TFAP2a, ZIC3 and MSX2 mRNA levels were significantly lower in both RIV lines than in H9 cells (Fig. 2.6A). In contrast, the mesodermal genes T/BRA, TBX6, MIXL1 and MIER1 were expressed in the RIV9 line at 2-, 37-, 2- and 18-fold the levels found in H9 cells, respectively (Fig. 2.6A). Contrastingly, RIV4 cultures had lower levels than RIV9 of most of the neural crest and mesodermal genes, but highly expressed SOX1 by 14-fold over the H9 line (Appendix Fig. 1.2.4) potentially suggesting that their differentiation was halted at a neuroepithelial state [57]. H9 cultures with a CRISPR/Cas9-mediated disruption of T/BRA did not exhibit a reduction in calcification, while disruption of the same gene in RIV9 cells lessened the degree of calcification (Fig. 2.6B). This result potentially suggested that osteogenesis occurred independently of T/BRA in H9 cells but was dependent on a mesodermal transition state in RIV9 cells.

Given that iPSCs have been reported to bear epigenetic marks from their cell type of origin [19] and given the high correlation between PAX7 and TWIST1 expression with the calcification yield in the H9 line over the RIV9 line we next examined the methylation status of both promoters. Methylation specific PCR supported the notion that the PAX7
promoter region was hypomethylated in the H9 cells, while it was methylated in the RIV9 hiPSCs (Fig 2.6C). This methylation pattern suggested that the activation of this locus was hindered in the RIV9 cells, which was reflected in the PAX7 mRNA levels. In turn, the evaluation of the TWIST1 promoter methylation status, a gene expressed by neural crest
cells once they have committed to a mesenchymal fate, revealed high methylation of the \textit{TWIST1} promoter in the H9 line, whereas \textit{TWIST1} was less methylated in the RIV9 cell line (Fig. 2.6C). \textit{In vivo}, \textit{TWIST1} mediates cranial bone progenitor specification [58], and is highly expressed in mesenchymal cells and then down-regulated for further differentiation to occur [59, 60]. The detected methylation status on the \textit{TWIST1} promoter suggested that in the RIV9 cells \textit{TWIST1} was turned on prematurely, thwarting its proper activation pattern during differentiation. Indeed, already in undifferentiated RIV9 cells \textit{TWIST1} was expressed 2.2–fold higher over the H9 hESCs (Fig. 2.6D) and increased with faster kinetics.

Next, we additionally analyzed the \textit{PAX7} and \textit{TWIST1} methylation status over a selection of different hESC and hiPSC lines and found differential methylation states that did not correlate with hESC or hiPSC identity (Appendix Fig. 1.2.5). However, the methylation status of these two promoters in the undifferentiated state did seem to be predictive of the osteogenic differentiation capacity of the respective lines. Moreover, the methylation status also predicted the ability of two karyotypically abnormal RIV lines, RIV1 and RIV7, independently of their karyotypic abnormalities (Appendix Fig. 1.2.5B, E). Together, these findings suggested that both \textit{PAX7} and \textit{TWIST1} methylation status may have a determining role in osteoblast development in PSCs.

\textit{RIV9 hiPSCs are able to predict skeletal teratogenesis despite their low osteogenic yield}

While a low osteogenic differentiation potential of a pluripotent stem cell line could be of concern for its clinical application in cell-based regenerative medicine, it may still
prove useful for the *in vitro* screening of putative skeletal toxicants despite its diminished osteogenic yield. To test this, we next examined the potential of the RIV9 line to accurately predict the toxicity of the strongly embryotoxic agent 5-Fluorouracil (5FU) [62] by assessing its inhibition of differentiation compared to its cytotoxicity [45]. The half-maximal inhibitory concentrations (ID₅₀) in the endpoint calcium content were not statistically different between the RIV9 and the H9 cells (P=0.090), neither were the half-

![Table and Graph](image)

**Figure 2.7.** Toxicity of 5-fluorouracil (5FU) tested with H9 and RIV9 hPSCs. (A) Table of half-maximal inhibitory doses for cytotoxicity (IC₅₀) as determined from concentration-response curves with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyterazolium bromide assay and differentiation (ID₅₀) with calcium assay. (B) Linear regression of RIV9 half-maximal inhibition of differentiation graphed against H9 half-maximal inhibition of differentiation. Both cell lines showed similar classification in predicting skeletal toxicity of 5FU. Abbreviations: 5FU, 5-fluorouracil; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyterazolium bromide.
maximal inhibitory concentrations of cytotoxicity (IC\textsubscript{50}) \((P=0.172)\) (Fig. 2.7A). Plotting the measured reduction in calcium content for every tested concentration in 5FU-treated osteogenic RIV9 cultures against the respective result found with the H9 cells (Fig. 2.7B) resulted in correlation coefficients close to 1, further demonstrating that RIV9 cells exhibit the same sensitivity to 5FU as H9 cells. These results potentially proved the usefulness of PSC osteogenic cultures in the prediction of skeletal toxicants, despite different differentiation kinetics or lower efficiencies.

**Discussion**

In line with previous studies, we show here that hPSC cell lines are generally capable of osteogenic differentiation. However, to our knowledge this is the first report that VD\textsubscript{3}, an osteogenic supplement that acts to trigger osteogenic gene expression and calcification in mouse and marmoset ESCs [6, 12], can also enhance human ESC osteogenic differentiation as well as induce osteogenic differentiation of human iPSCs. Notably, calcification levels were significantly lower in the two tested hiPSC lines than in the H9 hESC line. Where other studies have shown that osteoblasts can be derived from iPSCs when grown on scaffolds or with culture additives in general, most of these relied on qualitative von Kossa and Alizarin Red stains [8, 11]. Because only minimal quantitative analyses such as ALP and OCN were used in past studies [10], a thorough evaluation of the differences between hESCs and hiPSCs had not been performed [24, 63]. Using a broad range of techniques, including quantitative PCR, immunocytochemistry, and innovative video bioinformatics we show here systematically that although osteogenically
differentiating hiPSCs can form a mineralized matrix, the two hiPSC lines tested exhibited lower osteogenic efficiency than H9 hESCs by multiple decades. While we initially hypothesized that this difference had to do with the type of cell studied we soon found evidence that these differences in osteogenic yield were cell type independent.

In contrast to the results presented here, a recently published study on mouse ESCs and two mouse iPSC lines did not identify any differences in osteogenic yield based on global gene expression analysis [73]. However, some data in another previous study hints at a lower osteogenic yield in both mouse and human iPSC lines [64]. Potentially, these discrepancies could be either based on species-specific differences of how differentiation proceeds or, alternatively, on the fact that some of the tested lines were grown on feeder cells while others were not, which may affect their pluripotency states. Indeed, future studies should examine whether returning all cell lines to naïve pluripotency would equalize the methylation states of the key promoters analyzed here and thus level the osteogenic yield in all lines and potentially across species.

For now, the notably low differentiation yield in some hPSC lines may prove problematic in future applications for regenerative purposes. This is because large numbers of transplantable cells will be needed for any type of tissue repair [65, 66]. Hence, a low differentiation yield would render the already problematic scaleup even more labor-intensive. Of highest concern, however, is the fact there have so far been no investigations into the tissue origin of the osteoblasts that hPSCs generate. This is especially puzzling since it is well known that neural crest-derived frontal osteoblasts display a superior capacity to undergo osseous healing compared with calvarial osteoblasts of paraxial
mesoderm origin [67]. In a grafting study, Leucht and colleagues showed that neural crest-derived bone would graft and give rise to new bone regardless of whether they were placed into a mandibular or tibial defect [33]. In contrast, tibial bone placed in the mandibular defect, the mesodermal tibial cells made cartilage in the mandible instead of bone. Jheon and Schneider [34] have further suggested that bone of neural crest origin will graft better when placed with its bone tissue of origin.

We suggest here for the first time that in some hPSCs osteoblasts may arise through a different embryonic lineage than in others. Specifically, differential regulation of TWIST1 and PAX7 mRNA expression caused by differential promoter methylation may be the apparent cause for defective neural crest osteogenesis in the RIV9 and RIV4 cell lines, which is otherwise found in H9 cells.

The low levels of T/BRA and PAX7 expression in RIV4 hiPSCs potentially explains the decrease in RIV4 osteogenic yield, while positivity for SOX1 could indicate that most of the RIV4 cells specify to neuroepithelium [57], but then fail to differentiate further. In contrast, PAX7 and SOX10 expression and stage-appropriate downregulation of TWIST1 mRNA correlate with a neural crest origin of H9-derived osteoblasts. Interestingly, the RIV9 cells highly expressed T/BRA, TBX6, MIXL and MIER1 mRNA with decreased PAX7, ZIC1 and SOX1, indicating a preference for mesodermal osteogenesis in those cells. We suggest this because mesendodermal differentiation is typically associated with a ~90-fold increase in the expression of T/BRA over undifferentiated cells [68], while in our cultures it is up-regulated only 3.5-fold. Emerging evidence suggests that presence PAX7 may function to push cells towards a neural crest lineage [49, 50]. Our findings of PAX7
gene expression and methylation status between the different human pluripotent stem cell lines coupled with high expression of \textit{TFAP2a, ZIC1 and ZIC3} appears to support this notion. We show that H9 cells express higher levels of the \textit{PAX7} gene coupled with an unmethylated promoter region. Thus, the RIV9 line could exhibit silencing of this locus through DNA methylation regulation.

We also concentrated on \textit{TWIST1} as a candidate for assessing osteogenic potential because \textit{TWIST1} seems to regulate the commitment of mesenchymally fated cells to the osteogenic lineage. While high \textit{TWIST1} levels maintain mesenchymal stem cells in their unspecified state [60], silencing of \textit{TWIST1} enhanced matrix mineralization and osteoblast gene expression in C3H10T1/2 cells [59]. In cranial cells, loss of \textit{TWIST1} leads to the maintenance of an epithelial character and reversal of EMT [69, 70]. Here we have revealed a potential role of gene-specific hypomethylation in limiting osteogenic differentiation of the RIV9 line, which may have caused the early overexpression of \textit{TWIST1}.

One further indication for a possible alternative osteoblast origin between the H9 and the RIV9 cells was the kinetic calcification analysis that we performed. The video bioinformatic calcification data indicated that calcification arose quicker in the RIV9 line than in the H9 cells, validating findings by Nishikawa and colleagues, who had suggested a timely discrepancy between the appearance of neural crest and mesoderm derived mesenchymal cells, whereby the mesoderm derived mesenchymal progenitors appeared five days earlier than those derived from the neuroepithelium [57].

While the lower osteogenic output of some hPSC lines could mean that the development of an appropriate therapy for skeletal regeneration may require further
investigations, this lower yield does not seem to preclude their use in the identification and prediction of the embryotoxicity associated with exposure to a chemical compound. Researchers are utilizing hPSCs increasingly for developmental toxicity testing, however there is a lack of different tissue endpoints such as bone [71]. Furthermore, the capacity of hiPSCs as a screening tool has not been explored [72]. We show here that hiPSCs may be potentially useful in the \textit{in vitro} prediction of embryotoxicity even though a specific line may have a lower osteogenic yield.

\textbf{Conclusion}

In summary, our study demonstrates that different hPSC lines, including individual hiPSC clones, could have inherently different abilities to produce functional osteoblasts potentially based on the methylation marks placed on promotor regions important for lineage decisions. This could have an impact on the potential application domain of these cells. While low differentiation yields may be ameliorated by simple changes to the culturing protocol, for example EB-mediated differentiations or alternative supplements, future studies need to take into account the different capability of individual hPSC lines and characterize the process by which they lay down osteoblasts. Also, it will be important to investigate whether mesodermal-derived osteoprogenitors and hPSC-derived neural crest cells repair calvarial and long bone defects equally well.
References


38. Kuske B, Savkovic V, zur Nieden NI. Improved media compositions for the
differentiation of embryonic stem cells into osteoblasts and chondrocytes. Methods


40. Davis LA, Dienelt A, zur Nieden NI. Absorption-based assays for the analysis of

41. Majumdar S, Ramachandran S, Cerione RA. New insights into the GTP binding/GTP

42. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
quantitative PCR and the 2(-Delta C(T)) Method Methods 2001;25:402-408.

43. Leung AW, Murdoch B, Salem AF et al. WNT/β-catenin signaling mediates human
neural crest induction via a pre-neural border intermediate. Development.

44. Sung CO, Lee KW, Han S et al. Twist1 is up-regulated in gastric cancer-associated

45. Kuske B, Pulyanina PY, zur Nieden NI. Embryonic Stem Cell Test: Stem cell use in
predicting developmental cardiotoxicity and osteotoxicity. In: Harris C and Hansen JM,

46. zur Nieden NI, Price FD, Davis LA et al. Gene profiling on mixed embryonic stem cell
populations reveals a biphasic role for betacatenin in osteogenic differentiation. Mol
47. zur Nieden NI, Davis LA, Rancout DE. Monolayer cultivation of osteoprogenitors shortens duration of the embryonic stem cell test while reliably predicting developmental osteotoxicity. Toxicology 2010;277:66-73.


58. Yen HY, Ting MC, Maxson RE. Jagged1 functions downstream of Twist1 in the specification of the coronal suture and the formation of a boundary between osteogenic and non-osteogenic cells. Dev Biol 2010;347:258-270.


CHAPTER 3

Pluripotent Stem Cells as Tools to Assess Developmental Toxicity:
Diversity Instead of Consolidation

Nicole R.L. Sparks and Nicole I. zur Nieden

Introduction

The embryonic development of an organism represents a relatively short, yet vulnerable, span in its entire life cycle. Therefore, any teratogenic compound acting upon the developing organism during pregnancy may severely perturb development. In the early 1960s, the thalidomide disaster demonstrated the urgent need to identify and predict the developmental toxicity of putative toxicants before being released into the environment or for human use for the first time.

Nowadays, human populations are experiencing a rapid increase in exposure to potential environmental toxicants in the form of pharmaceutical drugs, commercial chemicals, industrial by-products, and wastes that are untested for safety. Most of these chemicals need to be assessed for their health risks to determine acceptable exposure levels. In vivo studies according to the Organization for Economic Co-operation and Development (OECD) guidelines are currently performed to provide such data. It is estimated that 100,000 existing chemicals circulate the market in the United States and that 2000 new substances enter it each year (Carpenter and Bushkin-Bedient, 2013; Locke and Bruce
Myers, 2010). Apart from the tremendous associated costs, in vivo testing of such a
magnitude of chemicals would require enormous amounts of animals and a great deal of
time. Clearly, in vivo testing methods would not be feasible to adequately test all current
and putative substances. Hence, it is crucial to develop alternative in vitro methods to
assess toxicity, especially teratogenicity, to test current and putative chemicals for their
impact on prenatal development while at the same time reduce the use of animals and cut
expenses.

Over the past 30 years, a number of in vitro approaches have been developed to
screen chemicals for their teratogenic effects and circumvent traditional in vivo methods.
In 2002, three of these in vitro embryotoxicity assays were validated according to standards
of the European Centre for the Validation of Alternative Methods (ECVAM): the
micromass assay (Umansky, 1966), the whole embryo culture assay (Cuthbertson and
Beck, 1990), and the embryonic stem cell test (EST) (Genschow et al., 2000, 2002).
However, micromass and whole embryo culture assay are labor intensive and still require
the killing of pregnant animals and/or embryos. The only in vitro mammalian-based
developmental toxicity test that does not sacrifice pregnant animals is the EST, as it uses
permanent cell lines, embryonic stem cells (ESCs), and 3T3 fibroblasts.
Embryonic Stem Cells

In recent years, scientific understanding of stem cells has greatly expanded the field of toxicology. ESCs are derived from the inner cell mass of the blastocyst-stage embryo (Figure 3.1). ESCs can maintain pluripotency for prolonged periods in vitro, have the ability to undergo sustained proliferation, and are able to differentiate and give rise to all three germ layers and thus all cells of the body (Vallier, Reynolds, and Pedersen, 2004). ESCs express OCT-3/4, NANOG, and SOX-2 (Chen and Daley, 2008) to maintain pluripotency, and the downregulation of these genes is associated with differentiation initiation (Rao and Orkin, 2006). The prolonged lifespan of ESCs is attributed to high telomerase activity, which is typically low in somatic cells (Thomson et al., 1998). Thus, ESCs have an unlimited proliferation capacity in vitro, which does not hold true for adult stem cells (Hiyama and Hiyama, 2007).

Figure 3.1. Embryonic stem cell derivation and differentiation. Totipotent cells (zygote) can form all types of cells including the organism and the trophoblast. Pluripotent embryonic stem cells are derived from the inner cell mass of the blastocyst-stage embryo and can form all three germ layers, but not the extra embryonic structures. http://www.musclesused.com/types-muscles/
For ESCs to differentiate toward a specific lineage, expression of pluripotency markers must be suppressed. In addition, chemical cues can be added to further push cells toward a particular lineage. ESCs progressing toward a particular lineage first produce a daughter cell and a progenitor cell, in an asymmetrical division (Sommer and Rao, 2002). Progenitor cells undergo a limited number of divisions (a distinction from ESCs) before terminally differentiating into a mature cell (Seaberg and van der Kooy, 2003). In a nonadherent environment, ESCs aggregate to form embryoid bodies (EBs), which comprise progenitor cells of various lineages. Owing to this ability to propagate themselves indefinitely and to mimic embryogenesis by producing a multitude of cell types, ESCs are a developmentally powerful tool for toxicological assays. For instance, cultures of spontaneously differentiated ESCs will contain contracting cardiomyocytes (Christoforou et al., 2008), a feature that is exploited in the classic EST.

**Embryonic Stem Cell Test**

The EST is the most promising mammalian *in vitro* assay to predict the embryotoxic potential of test chemicals. Many congenital abnormalities occur because of the adverse effects of deleterious agents on development processes, and as the EST predicts teratogenic potential during this growth period, it is the ideal test. It classifies substances into three classes of *in vivo* embryotoxicity (strong, weak, and not embryotoxic) (Scholz et al., 1999a; Genschow et al., 2002). Murine embryonic stem cells (mESCs) are utilized in the EST to assess the embryotoxic potential of the test substance as their *in vitro* differentiation recapitulates developmental processes and is characterized by gene
expression patterns indicative of in vivo cardiogenesis.

3T3 fibroblasts are also evaluated as they model the effect of the chemical on mature somatic cells (Figure 3.2). For the classification of the embryotoxic potential of the test compound into the above-named categories, three endpoints are determined: (i) cell differentiation: the concentration of the test compound that causes a 50% inhibition of the differentiation of ESCs into cardiomyocytes (ID$_{50}$); (ii) viability of immature cells: the concentration of the test compound resulting in a 50% decrease in the viability of ESCs [assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide) assay] (IC$_{50}$); and (iii) viability of mature somatic cells: the concentration of the test compound resulting in a 50% decrease in the viability of 3T3 fibroblasts (IC$_{50}$) (Scholz et al., 1999b). As previously mentioned, spontaneously differentiating EBs contain cell types of all three germ layers, amongst them beating cardiomyocytes. Indeed, the heart is the first organ to form in the developing embryo and

Figure 3.2. Embryonic stem cell test (EST). Overview of the approach and endpoints to assess the embryotoxic potential of a test chemical using mESCs and mouse fibroblasts.
contracting clusters of cardiomyocytes can be microscopically identified \textit{in vitro}, which suggested that cardiomyocytes are a suitable endpoint for the EST.

In addition, the EST is the only \textit{in vitro} test with a biostatistical prediction model that circumvents the use of animals to evaluate the embryotoxic potential of test compounds (Scholz \textit{et al.}, 1999a,b; Genschow \textit{et al.}, 2000, 2002, 2004). Using this prediction model, the National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) at the German Federal Institute for Risk Assessment coordinated ECVAM’s prevalidation and validation studies of 6 and 14 compounds, previously characterized by \textit{in vivo} data, respectively. The prevalidation study evaluated the prediction model designed for the EST, and based on those results, the prediction model was reevaluated with the remaining 14 test substances in the validation study (Spielmann \textit{et al.}, 2006). The validation study resulted in a 78\% correct classification of the 20 test compounds (Genschow \textit{et al.}, 2000, 2002, 2004). In addition, the test displayed 100\% predictivity for strong embryotoxic chemicals (Spielmann \textit{et al.}, 2006). The validation study comprises an intra- and interlaboratory exploration using six strong embryotoxic substances, seven weak substances, and seven nonembryotoxic substances under blind conditions in four laboratories (Spielmann \textit{et al.}, 2006). All four laboratories showed 100\% predictivity and 85\% precision of strong embryotoxic chemicals. Moreover, weak and nonembryotoxic correct classification of chemicals exhibited 83 and 75\%, respectively. Furthermore, there was a correlation between \textit{in vivo} and \textit{in vitro} classification in the four laboratories of 85, 83, 81, and 68\%. These numbers show that the test could not decipher between weak and nonembryotoxic compounds, despite being successful in identifying
strong embryotoxic chemicals. Moreover, the test could not detect compounds whose metabolites act as embryotoxicants; however, the overall positive results gave the EST the green light to be considered for regulatory uses (Genschow et al., 2002).

With the validation from ECVAM, some pharmaceutical and chemical companies employed the EST and found it useful for early stages of product development. When originally invented, the EST was designed for access to all laboratories, including pharmaceutical and chemical companies, with a standard, validated set of procedures. A decade later, improvements were clearly needed for the EST. Pfizer utilized ECVAM’s validated EST with compounds used to validate the EST and in-house compounds (Paquette et al., 2008). Their study showed that the test was robust and able to be used in other laboratories, however needed improvements. In pursuit of developing a standardized EST for all laboratories, ECVAM held a workshop in 2003 to address the future of the test. Key toxicologists determined that testing 20 compounds was not sufficient, and the choice of chemicals needed to be expanded for validating the test (Spielmann et al., 2006). Additionally, molecular endpoints and additional differentiation pathways, such as neural and osteogenic, needed to be included. Furthermore, it was suggested that the successful use of human embryonic stem cells (hESCs) in embryotoxicity screens would abrogate interspecies differences and was more reliable to predict human effects, while still reducing the number of laboratory animals used. Thus, instead of further validation studies for standard use, more in vitro models for prediction were developed.

These modifications to the EST and new in vitro models were done in-house, which created diversity amongst screening methods instead of further consolidating the method.
Developmental toxicology must assess the efficacy of the models to produce a high throughput coherent set of procedures for all to adapt to. However, owing to these in-house developments, the new methods, some of which are reviewed later, were not tested for inter- and intra-laboratory capability and not validated although promising.

### 3.1 Improvements to the Embryonic Stem Cell Test

Although it was established early on that ESCs are a powerful and valuable tool to examine the embryotoxic potential of a chemical, the originally tested cardiogenic endpoint is problematic. The readout of the classic EST is based on the presence or absence of beating cardiomyocytes and requires skilled personnel to microscopically detect such cells. In addition to being a technically challenging endpoint, cardiogenesis may also not be a valid endpoint for all teratogens. For example, a variety of developmental toxicants result in limb, skeletal, or CNS (central nervous system) malformations (zur Nieden et al., 2004). It was therefore deemed to be beneficial to differentiate ESCs into other lineages that can be easily tested with the EST to identify developmental toxicity in other endpoint organs (zur Nieden et al., 2004). For instance, the EST is one of few tests available to study the effects of agents on skeletal development in vitro (zur Nieden et al., 2010a, 2010b; zur Nieden and Baumgartner, 2010). Our group has established a method that abrogates the need for expensive molecular assays and utilizes morphometric image analysis to assess developmental osteotoxicity (zur Nieden et al., 2010a). We have used the mouse-based assay to determine developmental osteotoxicity in pharmaceutical compounds and have
also investigated developmental osteotoxicity of chloride derivatives (zur Nieden and Baumgartner, 2010).

In addition to the osteogenic endpoint, neural, chondrogenic, and endothelial endpoints were also developed to assess the embryotoxic potential on such cells (zur Nieden et al., 2004; Festag et al., 2007a). This ability to test additional differentiation pathways is due to advances in stem cell biology that allow for the directed differentiation of the cells into specific lineages with high yield (zur Nieden et al., 2003, 2005; Buttery et al., 2001; Festag et al., 2007b). Interestingly, these studies highlight concentration- and tissue-specific effects of compounds. For example, at a specific concentration, retinoic acid, a classified strong teratogen, caused a 400% increase in osteoblast marker gene expression, a 50% decrease in a chondrocyte specific mRNA, and no disruption of neural or cardiomyocyte gene expression. These intriguing results underscore the importance of testing multiple differentiation pathways.

In addition to developing screens for multiple diverse differentiation pathways, laboratories made improvements to the EST by establishing molecular endpoint analyses that could enhance the predictivity of the test. Bremer et al. (1999) were the first to use transgenic mESCs, in which green fluorescent protein (GFP) linked to a cardiac specific promoter was used as a toxicological endpoint by quantifying the number of GFP positive cells in cultures treated with chemicals known to affect in vivo development with flow cytometry. At the same time, flow cytometric methods provide the ability of high-throughput screening and are a more robust and reproducible screening system. Seiler et al. (2002) also developed a quicker and more reproducible screen by discovering that the
flow-cytometry-based detection of tissue-specific markers in compound-treated mESCs could accurately predict teratogenicity. In addition to protein expression, we reported that changes in marker gene expression in treated cells as quantified by RT-PCR (reverse transcription polymerase chain reaction) are predictive of the teratogenic potential of the compound. Therefore, the analysis of gene expression is a promising tool to study developmental toxicology because embryogenesis is aided by the changes in the regulation of genes. An advantage of determining which genes are affected by specific compounds is that it can lead to further elucidation of the underlying mechanisms of the teratogenic effect. Aside from tissue-specific protein and gene expression, the spontaneous extracellular field potential of differentiating cardiomyocytes measured with a microelectrode array system was found to be inhibited by embryotoxic chemicals (Koseki et al., 2010). Such promising studies underline the fact that it is worthwhile to include additional endpoints that enhance the predictivity of the EST.

Other improvements to the EST were attempts to reduce test duration. Although still quicker than an in vivo experiment, the first beating cardiomyocytes emerge at 9–10 days after in vitro culture, which does not facilitate the high throughput demands of testing the increased number of toxicants in the environment. It is necessary to develop an in vitro screening assay that reduces the time it takes to have the results in hand. Cardiac protein and gene expression endpoints mentioned above not only served the purpose of increasing predictivity but also allowed earlier detection of teratogenic effects on cardiogenesis. As such, quantitative PCR analysis of cardiac-specific myosin heavy chain (MHC) expression reduced the test to 8 days and still accurately assessed chemicals in
accordance with their *in vivo* potential (zur Nieden et al., 2001). In addition, the FACS-EST (fluorescence-activated cell sorting embryonic stem cell test) produced the same sensitivity as the validated EST but reduced the length of time of the assay to 7 days (Buesen et al., 2009; Seiler et al., 2004). While one would expect the appearance of mRNAs before the appearance of protein, the discrepancy in timing between these two studies may potentially be explained by the different serum lots used for the differentiation of the ESCs. Similar attempts were made by our group to shorten the duration of skeletal endpoint differentiations (zur Nieden et al., 2010b). While we were successful in assessing osteotoxicity at the level of mRNA expression of bone marker genes, these endpoints could not be analyzed until 30 days of osteogenic differentiation. To shorten the assay, we have made significant improvements to the culturing method and can now assess the teratogenic

![Figure 3.3](image-url) **Figure 3.3.** Human ESC osteogenic induction. Osteogenic differentiation is initiated after the cultures become confluent (designated d0) with Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS). On day 5, 1,25(0H)2 vitamin D3 (VD3), β-glycerophosphate (GP), and ascorbic acid (AA) are added to the medium to direct cells toward the osteogenic lineage. The presence of calcification is measurable on day 14, and by day 30, the culture is predominantly osteoblasts.
potential of a chemical in 14 days (zur Nieden et al., 2010b). We currently use a variation of this method for human pluripotent stem cell induction to osteoblasts and assessment of toxicants (Figure 3.3).

### 3.2 Beyond the Embryonic Stem Cell Test

With lack of consensus on which *in vitro* tests or endpoints are suitable for determining the embryotoxic potential of a compound, more developmental toxicity screens are being developed. For instance, the adherent cell differentiation and cytotoxicity (ACDC) assay is a murine-ESC-based model designed to (i) evaluate the effects of chemical exposure on stem cell number and differentiation using a single culture technique, (ii) quantify markers of cell number and cardiomyocyte differentiation, and (iii) provide an assessment of multiple differentiation markers (Barrier et al., 2011). The model abrogates the use of the EB-based differentiation system for an adherent method, which simplifies the culturing technique and is completed in 9 days. An advantage of this assay is the way cell number and cardiomyocyte differentiation are assessed; both are done after fixation on the same cells in the same culture wells. Cell number and MHC protein were both measured using the in-cell western protocol developed to use with the Li-Cor Odyssey infrared imaging system. This system measures two endpoints in each well with two infrared channels at 700 and 800 nm. In this study, Barrier *et al.* (2011) used the 700-nm channel to quantify the relative cell number and the 800-nm channel to detect the MHC signal. This assay is beneficial in that it simplifies culture techniques and can assess two endpoints in one vessel—creating a high throughput assay that has the potential of full automation.
(Barrier et al., 2011). Although promising, this assay does not include a prediction model such as the EST.

More than 90% of chemicals that enter the body are metabolized in the liver into other forms, metabolites, which are a potential culprit that skews development (Wilkinson, 2005; Kleinstruer et al., 2011). Primary rat hepatocytes have been utilized to metabolize the compound in vitro before analysis. However, these cells are difficult to maintain and are plagued by interspecies variation (Greenhough, Medine, and Hay, 2010). Furthermore, cells are typically harvested from rats, which would not reduce animal use. Other currently available systems for mimicking liver metabolism include the use of S9 liver extracts, transgenic cell lines, hepatocyte-like cell monolayers, and 3D organotypic cultures (Adler et al., 2011; Esch, King, and Shuler, 2011; Giri et al., 2011; Landsiedel et al., 2011). Pluripotent stem cell research has enabled the ability to derive hepatocyte-like cells from hESCs; however, the yield and efficiency are low and gene expression profiles vary (Greenhough, Medine, and Hay, 2010). With a clear need for metabolism models, North America and the European Union have created virtual liver initiatives. The US EPA (Environmental Protection Agency) Virtual Liver Project (Environmental Protection Agency (EPA), 2009) uses mathematical models to predict liver toxicity based on molecular targets, signaling pathways, and toxicity at the organ and organismal levels.

Similar to interspecies differences in the ability to metabolize substances, individuals too differ in their ability to metabolize chemicals (Xie et al., 2001), making it difficult to reliably predict toxicity using cells derived from a single donor (Greenhough, Medine, and Hay, 2010). Here, the development of induced pluripotent stem cells (iPSCs) has
revolutionized stem-cell-based toxicity screening. iPSCs are generated by the reprogramming of somatic cells through the introduction of four key transcription factors: Sox-2, Klf-4, Oct4, and c-Myc (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). These cells possess a number of advantages over hESCs, including patient-specific cell-based drug testing and are free from the ethical concerns associated with the use of blastocysts. The creation of liver toxicity screening technologies utilizing iPSC-derived hepatocyte-like cells would allow investigation into the effects of single nucleotide polymorphisms on drug metabolism and toxicity (Greenhough, Medine, and Hay, 2010).

**Model Organism**

Murine ESCs are able to recapitulate hallmarks of embryogenesis and murine-ESC-based embryotoxicity assays are thus a great tool to elucidate mechanisms of developmental toxicity. However, mouse models are not able to entirely mimic human development. Moreover, culturing methods and expression of certain genes differ between murine and human ESCs. For instance, human ESCs form flat colonies, do not survive as single cells, and require basic fibroblast growth factor (bFGF) in the culture medium to maintain an undifferentiated state, whereas murine ESCs have three-dimensional colonies, survive as single cells, and leukemia inhibitory factor is added to culture medium. These differences limit the transferability of predictions for human use made based on mouse cells. Indeed, *in vivo* developmental toxicity studies have shown high interspecies differences, despite the usefulness of the murine-based EST (Hurtt, Cappon, and Browning, 2003). Specifically, thalidomide has shown no teratogenic effects in mice, but causes
profound human congenital malformations (Gilbert, 2003; Fratta, Sigg, and Maiorana, 1965; Brent and Holmes, 1988).

Therefore, integrating human stem cells into developmental toxicity assays is necessary to accurately account for the human response to putative toxicants. In addition, these cells are highly attractive to the pharmaceutical industry in facilitating drug discovery development.

As a first step, Buzanska et al. (2009) utilized a human neural stem cell line derived from umbilical cord blood (HUCB-NSC) as a model to test developmental neurotoxicity. The study showed that differently staged HUCB-NSCs were able to discriminate between neurotoxic and non-neurotoxic chemicals. The endpoints (cell viability, proliferation, apoptosis, and expression of cell-specific markers) were assessed 48h after exposure to test chemicals of early and late stages of HUCB-NSC. Cell viability was measured by the live/dead viability/cytotoxicity (LDVC) assay, MTT assay, or the resazurin reduction assay. Nuclear chromatin staining and immunocytochemical labeling for stage-specific markers were used to determine cell proliferation, apoptosis, and neural differentiation. The LDVC assay allowed for the rapid discrimination of live and dead cells microscopically or with flow cytometric methods. The MTT and resazurin reduction assays are both colorimetric assays that rely on the ability of mitochondria to reduce MTT or resazurin, respectively, to measure viable cells. The downside of such assays is that they are not true viability tests because they measure mitochondrial potential, which could also be altered due to changes in cellular metabolism. Furthermore, apoptotic cell identification
could benefit from additional endpoints, such as caspase-3 quantification, to support chromatin condensation data.

Although some of the endpoint assays in the HUCB-NSC study could clearly be improved, an advantage of their approach was the usage of human cells. Advantageous from the standpoint of choice of test species, their approach differentiates neural progenitors as opposed to less committed cells and therefore evaluates particular stages of development only. In 2011, Colleoni et al. (2011) developed an alternative approach to assess neural teratogenicity using pluripotent human ESCs. The group differentiated human ESCs into neural rosettes, which represent neural plate and neural tube development, and exposed the cells to retinoic acid.

This treatment would induce neural tube defects, which is one of the most common birth defects, affecting nearly 0.5–2 newborns per 1000 (Colleoni et al., 2011). Their microarray analysis and qPCR data suggested dose-dependent misregulation of genes involved in neural tube formation and closure, such as the homeobox family genes and Otx2. Furthermore, this in vitro modulation of neurulation markers in response to retinoic acid correlated with gene expression responses associated with in vivo retinoic acid exposure. Despite being a first promising step toward a predictive human-based in vitro model, this approach requires further validation of other teratogenic chemicals and lacks a prediction model to classify substances such as the one used in the classic EST.

In a similar approach, West et al. (2010) developed a hESC-based model that used metabolomics to detect small molecule changes in response to teratogen exposure, a concept that was first introduced by Cezar et al. (2007). In drug treated and untreated cells,
the group reported significant differences in metabolite abundances, with asymmetric dimethylarginine (ADMA) being the most predictive of teratogenicity, particularly neural tube closure. The discovered alteration of ADMA upon teratogen exposure suggests that it is a potential biomarker for neural tube defects when modified. However, further exploration is needed to detect biomarkers in toxicity of differentiation pathways. Novel approaches such as this will potentially elucidate the underlying mechanisms of developmental toxicity.

**Conclusion**

There are many demands in the field of developmental and predictive toxicology to create an *in vitro* system that meets the standards of academic and industrial toxicity screening. Although *in vitro* screening methods in developmental toxicology have tremendously advanced, there is still a lack of agreement on what constitutes a developmental toxicant and how these characteristics such as physiological endpoints, maternal toxicity, dose magnitude, and duration can be translated into, or derived from the *in vitro* model (Castoldi *et al.*, 2008). The EST is the most promising validated *in vitro* developmental toxicity model, though its endpoints as well as the use of murine cells limit the utility of the test. These downfalls have created a frenzy of improvements and have led to new *in vitro* approaches (some including hESC models). New *in vitro* efforts would benefit the EST if toxicologists focused on standardizing a set of assays to assess developmental toxicity instead of creating diversity amongst the field.
References


37. Seiler A, Visan A, Pohl I, Genschow E, Buesen R, Spielmann H. Improving the embryonic stem cell test (EST) by establishing molecular endpoints of tissue specific


53. zur Nieden NI, Davis LA, Rancourt DE. Monolayer cultivation of osteoprogenitors shortens duration of the embryonic stem cell test while reliably predicting developmental osteotoxicity. Toxicology 2010b;277(1–3): 66–73.


CHAPTER 4

Differential predictivity of human pluripotent stem cell lines in skeletogenic developmental toxicity assays correlates with their innate differentiation bias towards neural crest or mesodermal osteogenesis

Nicole Renee Lee Sparks, Joseph Madrid, Riley Bottom, Nicole Isolde zur Nieden

Abstract

87.5% of registered human birth defects entail malformations of the skeleton, either in the craniofacial region or the limbs. A major preventable risk for the development of such deformities is chemical or drug exposure during pregnancy. However, unwanted chemical exposure may be prevented only if sufficient information on the developmental toxicity of such chemicals were available. The embryonic stem cell test (EST) comprehensively determines the potential of chemical entities to qualify as developmental toxicants based on multiple in vitro endpoints. These include the assessment of differentiation inhibition and the cytotoxic effect of the chemical in murine pluripotent mouse embryonic stem cells (ESCs) contrasted to the cytotoxic effect that this chemical elicits in fibroblasts. Due to the pluripotent nature of the ESCs used in this assay, the EST has been validated for multiple tissue endpoints including bone. However, potential interspecies variations in responses to chemicals raise concern about the efficacy of the rodent model to predict safe levels of compound exposure for humans. Thus, improvements
of the EST have focused on human-based modeling. Our present study demonstrates the promise of human pluripotent stem cells for in vitro developmental toxicity assays to uncover differentiation defects in the osteogenic lineage. First, our findings suggest that an assay based on human H9 ESCs can classify test compounds correctly, even for such compounds that classify incorrectly in the murine EST. Secondly, osteogenically differentiated H9 ESCs were more sensitive to chemicals known to negatively affect the developing neural crest, one of the two origins of bone tissue, than RIV9 human induced pluripotent stem cells (hiPSCs). This differential sensitivity correlated well with cell-line specific propensities to differentiate into neural crest or mesodermal osteoblasts. Accordingly, analysis of TWIST1 mRNA, a gene primarily expressed in neural crest-derived osteogenic precursors, showed higher predictivity in the H9 cells than the RIV9 cells. We conclude from our results that the described test system using human pluripotent stem cells provides an accurate assay to identify and predict the potency of chemicals to elicit skeletogenic developmental toxicity that is biologically significant for humans.

Introduction

The etiology of major musculoskeletal birth defects remains largely unknown but plays a large role in public health concerns. Musculoskeletal birth defects that affect skeletal tissues are frequent (Parker et al., 2010) and impose life-long health consequences to the affected individuals and their families. Musculoskeletal anomalies include cleft lip and/or palate, oculo-auriculo-vertebral syndrome, limb reduction, and skeletal dysplasias. Cleft lip/cleft palate, a congenital abnormality where the tissues of the upper lip or palate
do not fuse, occurs in 2-10/10,000 births (Burton, 2008). Limb reductions has an incidence of 2.5-7.06 per 10,000 births (Burton, 2008). Skeletal dysplasias, disorders that affect the development of the bone and cartilage, consists of hundreds of different classifications with an incidence of 2.1-2.3/10,000 births (Burton, 2008; Krakow and Rimoin, 2010). It is also plausible that these incidences could be higher because many anomalies do not manifest until later in childhood.

While genetic factors can be an underlying cause for some of these birth defects, others stem from involuntary environmental and chemical exposure of the fetus while developing in utero. To reduce the incidence rate of preventable birth defects, it is thus of utmost importance to identify chemicals that contribute to musculoskeletal maldevelopment. Stem-cell-based assays, which are also equipped to eliminate the use of animals, are gaining attention for this purpose. Among them, the Embryonic Stem Cell Test (EST) is the most advanced (Genschow et al., 2000; Pulyanina and zur Nieden, 2012; Kameoka et al., 2014).

It has been almost 25 years since Spielmann and colleagues introduced the mouse cell-based EST to assess the embryotoxic potential of chemical entities or blends in vitro (Heuer et al., 1993; Scholz et al., 1999a; reviewed in Seiler and Spielmann, 2011). Historically, this test utilized three endpoints. Mouse fibroblasts are exposed to a compound to assess maternal effects of the tested chemical. Any noted cytotoxic effect is then contrasted to the effect of the chemical on pluripotent mouse embryonic stem cell (ESC) viability and differentiation propensity (Genschow et al., 2000; Scholz et al., 1999b). While classically evaluating the inhibition of cardiac differentiation, a repertoire of
osteogenic, chondrogenic, endothelial, and neural differentiation was later added to test for cell lineage specific effects (de Jong et al., 2014; Festag et al., 2007a; 2007b; Hayess et al., 2013; Stummann et al., 2009; zur Nieden et al., 2004; 2010). This addition of tissue endpoints in combination with molecular endpoints seemed necessary to improve the predictivity of the EST (Koseki et al., 2010; Schenk et al., 2010; Seiler et al., 2004; van Dartel and Piersma, 2011). For example, in comparing the effects of compounds in the osteogenic and cardiogenic mouse EST, de Jong et al. (2014) showed the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on osteoblast, but not on cardiomyocyte differentiation.

Although highly predictive in comparison to other routinely used *in vitro* embryotoxicity assays (Scholz et al., 1999b; Genschow et al., 2002), the mouse-based EST still bares the risk of providing false negative or positive results based on potentially varied adverse outcome pathways in human versus the mouse. Changing the test species to non-human primate cells yielded differential responses between cells from rhesus monkey, marmoset monkey, and mouse, with no obvious dependency on chemical classes (Walker et al., 2014), supporting this hypothesis. Consequently, determining a safe exposure level for human use may be difficult for certain chemicals due to variations in response between different species, implying that human ESCs may be the most promising model to be used in such assays.

Indeed, commercially available mesenchymal stem cells derived from a human ESC line have been evaluated for predicting skeletogenic developmental toxicity (Sittner et al., 2016). The utilization of mineralization defects and their comparison with
cytotoxicity of chemicals to assess exposure, represented a step in the right direction. The study did not include the comparison to cytotoxicity on somatic cells. More importantly, a disadvantage of the use of mesenchymal stem cells is that, while they represent a source for osteoblasts, they themselves may arise from different lineages during embryogenesis: neural crest or mesodermal cells. The commitment of neural crest and mesodermal cells may be affected differently by chemicals, which may be missed when assaying osteogenesis only from the vantage point of a mesenchymal cell.

Here, we evaluated the ability of a comprehensive human-based EST that analyzes all three known mouse EST endpoints to predict skeletal embryotoxicity based on full osteogenic lineage differentiation from undifferentiated hESCs. In addition, we expanded our assessment to a line of human induced pluripotent stem cells (hiPSCs), a source of pluripotent stem cells artificially created from somatic cells (Takahashi et al., 2007). Together, our results illustrate the limitations of the mouse-based EST and emphasize the effectiveness of human-based in vitro models provided appropriate cell source/marker analysis combinations are used.

**Materials and methods**

*Fibroblast culture*

Human foreskin fibroblasts (HFFs) were graciously provided by Dr. Derrick Rancourt (University of Calgary, Canada) at passage 1. The cells were maintained in high glucose L-glutamine Dulbecco’s modified Eagle’s medium (DMEM, Corning) with 10% fetal bovine serum (FBS, Atlanta), 1% non-essential amino acids (NEAA, Gibco), and
0.5% penicillin/streptomycin (10,000 units/10,000 units, Gibco). Murine 3T3 fibroblasts (American Type Culture Collection, Rockville, MD, USA) were seeded on gelatin coated culture plates and cultured in high glucose L-glutamine DMEM supplemented with 15% FBS and 1% penicillin/streptomycin (10,000 units/10,000 units).

*Culture of embryonic and induced pluripotent stem cells*

Cells of the human H9 ESC line were acquired from WiCell (WiCell Research Institute). RIV9 hiPSC cells were previously generated from foreskin fibroblasts by retroviral integration of *OCT4*, *KLF4* and *SOX2* at the University of California Riverside’s Stem Cell Core Facility (Chatterjee et al., 2011; Torrez et al., 2012; Sparks et al., 2018). All human pluripotent cells were cultured on Matrigel (BD Biosciences) treated dishes and maintained as feeder-free cultures in mTeSR® media (Stem Cell Technologies) in 5% CO₂ and at 37°C. Pluripotent colonies were passaged every 5 days upon reaching 70% confluency by dissociating cells with accutase and a cell scraper.

Murine D3 ESCs (American Type Culture Collection, Rockville, MD, USA) were expanded in high glucose DMEM containing L-glutamine, 15% FBS (selected batch), 1% NEAA, and 1% penicillin/streptomycin (10,000 units/10,000 units), 0.1 mM 2-mercaptoethanol (Sigma), and 1000 U LIF/ml (Millipore). Cells were sub-cultured every 2–3 days with 0.25% Trypsin-EDTA (Life Technologies).
Osteogenic differentiation

Murine ESCs were induced to differentiate via aggregation into embryoid bodies and hanging drops at 750 cells/drop, in the presence of control differentiation medium (CDM, mouse ESC maintenance medium without LIF) (zur Nieden et al., 2003). Differentiating cells were replated on day 5 as a single cell suspension at a concentration of 50,000 cells/cm² (zur Nieden et al., 2010a).

Confluent pluripotent human stem cell colonies previously maintained in mTeSR remained in their culture vessel (designated day 0), and the media was changed to CDM. On day 5 of differentiation, cells from all species received osteogenic differentiation medium containing the induction factors β-glycerophosphate (10 mM), ascorbic acid (25 μg/ml), and 1α,25-(OH)₂ vitamin D₃ (5 × 10⁻⁸ M) in CDM.

Histochemical analysis

Cells were washed with 1X PBS and fixed in 4% paraformaldehyde at 4°C for 1 hr on day 30 of differentiation. For von Kossa stain, fixed samples were overlaid with silver nitrate solution (Ricca Chemical Company) and illuminated under an intense light source for 1 hr. Un-reacted silver was removed with 5% sodium thiosulfate (Red Bird Service) for 2 minutes. Calcium-rich areas were also visualized with 2% w/v Alizarin Red S (Sigma-Aldrich, pH 4.1-4.3). Cells were washed with PBS and overlaid with the Alizarin Red S solution for 5 minutes to form an Alizarin Red S-calcium complex. Cultures were washed 3X with water followed by ascending alcohol washes (70%, 80%, 90%, 100% ethanol).
**Immunocytochemistry**

Cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 30 min at 4°C. Membranes were permeabilized for intracellular antigen staining with 0.1% Triton-X 100 (Sigma) for 15 min and then incubated overnight at 4°C in PBS/10% FBS containing either OCN (Abcam, ab1857), SOX10 (Santa Cruz, SC17342), TWIST1 (Abcam, ab50887) and/or PAX7 (mIgG1, Developmental Studies Hybridoma Bank) antibody. Cells were then washed and incubated in the appropriate secondary antibody (AlexaFluor conjugated) for 2h and observed on a Nikon fluorescent microscope.

**Test compounds**

The chemicals 5-fluorouracil (5FU), all-trans-Retinoic acid (atRA), and penicillin G sodium salt (PenG) were used in the original EST validation study (Genschow et al., 2004) and were used here as positive and negative control model compounds. Exposure to 13-cis-Retinoic acid (13cisRA), a known human teratogen with lesser effects in the mouse (Nau, 2001), was chosen for demonstrating potentially variable outcomes depending on cell lines examined. Cyclopamine, methoxyacetic acid (MAA), triadimefon, and triadimenol were selected for their known inhibitory effects on osteoblasts generated from the neural crest and/or the mesoderm (Jeong et al., 2004; Brown et al., 1984; Papis et al., 2006; Zimmer et al., 2012). All test compounds were purchased from Sigma-Aldrich and either dissolved in DMSO or cell culture medium, based on solubility of the compound (Buesen et al., 2009). Differentiation of ESCs was initiated as described above and cells were treated for 20 days of osteogenesis with seven or eight concentrations of test
compound. As per ECVAM standards, the maximum final concentration of any test chemical was 1000 µg/ml (Spielmann and Seiler, 2010). A solvent control containing the highest concentration of solvent was included to control for non-chemical specific effects. All compounds were replenished with each media change.

**Mitochondrial dehydrogenase activity assay**

Cellular survival in response to test compounds was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide (MTT, Spectrum Chemicals). Briefly, cells were incubated with MTT (0.5 mg/ml) at 37 °C for 2 hr. After the supernatant was removed, cells were gently rocked in pre-warmed MTT desorb solution (0.7% sodium dodecyl sulfate (SDS) in 2-propanol) for 15 min, and the optical density of the solution was read at 570 nm in an iMark™ microplate reader (Bio-Rad). As the generation of the blue formazan product is proportional to the dehydrogenase activity, a decrease in the absorbance at 570 nm provided a direct measurement of the number of viable cells. Activity was normalized to solvent only controls and resulting percentages were graphed along the tested concentration range to construct a concentration–response curve. The half-maximal inhibitory effect (IC_{50}) for each compound was subsequently established via linear interpolation of the curve.

**Calcium assay**

For quantification of extracellular matrix calcium, cells were harvested in radioimmunoprecipitation buffer (RIPA; 1XPBS, 1% NP-40, 0.5% sodium deoxycholate,
0.1% SDS, pH 7.4, and 1:100 protease inhibitors). Calcium deposition was determined based on calcium ions (Ca$^{2+}$) reacting with Arsenazo III (Genzyme, Canada) to form a purple Ca-Arsenazo III complex, which was measured at 655 nm. The concentration of total calcium in the sample was calculated based on a CaCl$_2$ standard and calcium content was normalized to the total protein content of the sample using the Lowry method (Bio-Rad) (Davis et al., 2011; Kuske et al., 2012) as we have also done previously when screening with mouse cells (zur Nieden et al., 2004; zur Nieden et al., 2010a, b). This normalization is important to truly assess the differentiation level of the cultures without interference from cytotoxic or proliferative events. Calcium content was then standardized to solvent only controls and concentration-response curves charted. The half-maximal inhibitory dose (ID$_{50}$Ca) for each compound was taken from linear interpolation of the curve.

**RNA isolation and quantitative PCR**

To analyze mRNA expression patterns upon differentiation or compound exposure cells were lysed in RA1 lysis buffer and total RNA was extracted according to the NucleoSpin RNA II protocol (Macherey Nagel). RNA was quantified with a NanoDrop® 1000 spectrophotometer (Thermo Scientific) at 260 nm. 25 ng of total RNA was used as a template for cDNA synthesis with a mastermix including 1X reaction buffer, 0.5 mM dNTPs, 20 U/µL RNase inhibitor, 0.8 U/µL reverse transcriptase, and 1 µM random primer. 25 ng of cDNA transcripts were used for quantitative polymerase chain reaction (qPCR) with SYBR green on the MyiQ cycler (BIO-RAD). The primers for **TWIST1** were 5′-
ACTGGCCTGCAAAACCATA-3’ (forward) and 5’-TGCATTTTACCATGGGTCCT-3’ (reverse). The primers for GAPDH were 5’-GAGTCAACGGATTTGGGTGT-3’ (forward) and 5’-TTGATTTTGGAGGGATCTCG-3’ (reverse). Reactions were setup for 10 minutes of denaturing at 94°C, followed by 40 cycles of denaturing at 94°C, and annealing/elongation at 60°C for 45 seconds. The n-fold expression in target samples was calculated with the ΔΔC_T method by standardizing C_T values to GAPDH expression (Livak and Schmittgen, 2001). Gene expression was normalized to solvent only controls and concentration-response curves charted. The half-maximal inhibitory dose (ID_{50} TWIST1) for each compound was taken from linear interpolation of the curve.

Statistical analysis

Data presented are averages of three independent experiments (qPCR) or five technical replicates ± standard deviation. Significance was determined by using a web-based one-way ANOVA and Tukey HSD post hoc test (http://faculty.vassar.edu/lowry/anova1u.html) or unpaired Student's t-test as appropriate.

Results

Comparison between predictivity for 13cisRA in mouse and human ESCs

To determine whether human cells would more accurately mimic phenotypes found after human exposure than mouse cells, we tested a selected set of four chemicals for which the outcome of in vivo exposures is adequately described. Penicillin G (PenG) was used as a non-embryotoxic chemical, 5-fluorouracil (5FU) as a strong embryotoxic chemical with
general cytotoxicity due to interference with DNA synthesis, and \textit{all-trans}-Retinoic acid \textit{atRA} because it has shown concentration-dependent toxicity on the osteogenic lineage (Scholz et al., 1999; Genschow et al., 2000; zur Nieden et al., 2004; zur Nieden et al., 2010). In addition, we included \textit{13cisRA}, which shows stronger exposure responses in humans than in rodents and the profound unwanted effects of retinoids on bone (Kraft et al., 1987; Nau, 2001; DiGiovanna, 2001).

Towards this goal, mouse and human ESCs were differentiated according to Fig. 4.1A and concomitantly exposed to the test chemical. Murine and non-human primate ESCs have been reported to differentiate into functional osteoblasts capable of mineralizing their extracellular matrix (ECM) (zur Nieden et al., 2003; Ding et al., 2012; Trettner et al., 2014; Sparks et al., 2018). Solvent controls exhibited the expected mineralization of the matrix (Fig. 4.1B), which comprises the incorporation of calcium visible as black deposit in bright field microscopy (Fig. 4.1C). In the location of the mineralized areas, Alizarin Red S and von Kossa stains positively confirmed deposition of calcium. The calcified dark areas of the ECM were also immuno-reactive to osteocalcin (Fig. 4.1C).

All chemicals were then tested in concentration response curves (Appendix Figs. 1.4.1 and 1.4.2) and half-maximal inhibitory concentrations for cell viability (IC50) and differentiation (ID50Ca) were established (Fig. 1D). In addition to the assessment of cell viability and differentiation inhibition on ESCs, the mouse EST compares the IC50 and ID50 to the cell viability of 3T3 fibroblast cells of the same test compound (Scholz et al., 1999), to represent the cytotoxic effect of the test chemical on the mother. For the inclusion
of this third endpoint in a human cell-based assay we measured cell viability on exposed human fibroblasts.

Figure 4.1. Human ESCs are more sensitive in predicting the developmental toxicity of 13cisRA, a known human teratogen, than mouse ESCs. (A) Schematic of protocols for osteogenic differentiation of mouse (D3) and human (H9) ESCs. (B) Calcium content in mouse and human ESC cultures differentiated per protocols in (A), either with the addition of the osteogenic factors Vitamin D3, ascorbic acid and β-glycerophosphate or without. (C) Photomicrographs of H9 hESC osteogenic cultures on day 30 of differentiation. Black deposits in brightfield images and positivity for von Kossa, Alizarin Red S and osteocalcin (OCN) indicate the presence of osteoblasts. Bar = 100 µm. (D) Mouse and human ESCs were exposed to listed compounds during differentiation. Calcium content was measured to determine the degree of osteogenic differentiation and mitochondrial dehydrogenase activity served as a measure of compound cytotoxicity. Human foreskin fibroblasts and mouse 3T3 fibroblasts were also exposed and assayed with the MTT assay. The listed half-maximal inhibitory concentrations (IC\(_{50}\) and ID\(_{50}\)) were determined from concentration response curves that were generated for all cell lines and compounds (see also supplemental figures 1 and 2) and are expressed in µg/mL. Mouse ESC and 3T3 fibroblast data for Pen G (penicillin G), 5FU (5-fluorouracil) and all-trans retinoic acid (atRA) taken from zur Nieden et al., 2004. (E) Correlation between half-maximal inhibitory doses found for cell viability (IC\(_{50}\)) and differentiation (ID\(_{50}\)Ca) with murine and human ESCs. (F) Classification of the test compounds per ECVAM’s biostatistical model. 5FU, 5-fluorouracil; atRA, all-trans Retinoic acid; hESC, human embryonic stem cell; hFF, human foreskin fibroblast; PenG, penicillin G.
The three endpoints were then summarized in a biostatistical model that allowed for the classification of the test compound into three embryotoxicity classes: non-, weak, and strong embryotoxic potential (Genschow et al., 2000, 2002). This biostatistical model classified the three control compounds similarly between species (Fig. 4.1E), despite different sensitivity ranges (compare Appendix Fig. 1.4.1). For the strong embryotoxicant 5FU, human ESC cell viability and osteoblast differentiation endpoints were over 100-fold more sensitive compared to the mouse endpoints. Conversely, murine ESCs were more sensitive towards the strong embryotoxicant atRA than human ESCs, a pattern reflected when human fibroblasts were tested. Nonetheless, 5FU and atRA were predicted to be strong embryotoxicants and PenG to be non-embryotoxic matching their in vivo embryotoxicity classification.

For 13cisRA, half-maximal inhibitory doses were also lower when tested with human cells (Fig. 4.1D, Appendix Figure 1.4.2). The biostatistical model classified 13cisRA as non-embryotoxic (class I) based on the sensitivity of the mouse cells, and as moderately embryotoxic based on the human ESCs (class II; Fig. 4.1D). This suggests in vitro species-species variability in sensitivity to a compound that was similarly also found to be more embryotoxic in vivo. Half-maximal inhibitory doses for cell viability and differentiation obtained from both mouse and human experiments were next charted against each other in a linear regression model (Fig. 4.1F). Both charts showed a low correlation between the murine and human ESC endpoints producing $R^2$ values of 0.52 and 0.57, respectively, again underscoring the differential sensitivity to toxicants between the species.
Figure 4.2. Differentiation and cell viability endpoints measured with RIV9 hiPSCs. (A) Mitochondrial dehydrogenase activity and (B) calcium content (mg Ca^{2+}/mg protein) in response to chemical exposure. Both n=5±SD, *p<0.05 One-Way ANOVA. (C) Linear regression analysis for tested compounds between mitochondrial dehydrogenase activity and calcium content measured with H9 hESCs and RIV9 hiPSCs. 5FU, 5-fluorouracil; aRA, all-trans retinoic acid; 13cisRA, 13-cis Retinoic Acid; hiPSC, human induced pluripotent stem cell; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyterazolium bromide; PenG, penicillin G.
Evaluating the predictivity of RIV9 hiPSCs

Human iPSCs hold great promise in embryotoxicity screening assays, because they are cells generated from the reprogramming of somatic cells lacking the ethical challenges that hESCs face. To assess the suitability of an hiPSC cell line in the prediction of chemical embryotoxicity, RIV9 hiPSCs were osteogenically differentiated and simultaneously exposed to all four of the test chemicals. With the exception of PenG, all compounds inhibited cell viability and calcium content (Fig. 4.2A, B).

To summarize the data, we next charted the results obtained with the H9 cells to those obtained with the RIV9 cells. The correlation between the IC$_{50}$ values was high with $R^2 = 0.998$ (Fig. 4.2C). A similar strong correlation ($R^2 = 0.97$), was observed between the ID$_{50}$Ca of the two cell lines. When used in the biostatistical prediction model, the compounds were classified similarly, independently of the human PSC line that was used (Table 4.1). Therefore, the similarity between the responses to the test compounds in the two cell lines suggested the usefulness of RIV9 hiPSCs for *in vitro* embryotoxic testing to identify and predict putative embryotoxic compounds based on this selected set of chemicals.

Assessment of chemicals with lineage specific action

Recent studies from our lab have suggested that the propensity of various human pluripotent stem cell lines for osteogenic differentiation correlated with the methylation pattern of promoters important for lineage specification while cells are in the undifferentiated state (Sparks et al., 2018). This seems to dictate how efficiently H9 and
RIV9 cells calcify (Fig. 4.3A). It also relates to the kind of bone that is specified. Many cranial bones are formed through intramembranous ossification, in which mesenchymal precursors created from neural crest cells condense and directly differentiate into osteoblasts. In contrast, most bones, from the mesoderm lineage, undergo endochondral ossification, which requires a cartilage precursor to provide the framework for
vascularization and osteoblast calcification (Regard et al., 2012). In H9 cells, antibody stains on 4 and 9-day old cultures using PAX7, SOX10, and TWIST1 antibodies show sequential expression of neural plate border specifier proteins, neural crest specifier proteins, and proteins associated with neural crest migration. In RIV9 cells these proteins were expressed to a lesser degree. Instead, the mesodermal protein, TBRA, (Herrmann et al., 1990; Herrmann, 1991) was enhanced. Quantitative mRNA expression for mesoderm and neural crest-associated genes also revealed that RIV9 cells significantly expressed mesoderm-associated genes over the H9 cells but expressed neural plate border specifier genes and neural crest specifiers at a significantly lower level. Additionally, H9 cells displayed a higher potential to generate neural crest cells when taken through a protocol that will directly induce this type of progenitor cell (Leung et al., 2016) (Fig. 4.3D). In line with our previous study, these data suggested that H9 cells display a higher propensity to generate neural crest osteoblasts (Sparks et al., 2018).

Thus, we hypothesized that the H9 cells could potentially be more sensitive to chemicals that specifically target the development of bone from the neural crest than the RIV9 cells. To test this hypothesis, both cell lines were exposed to compounds with known in vivo toxicity to the neural crest: cyclopamine, methoxyacetic acid, triademefon and triademenol (Scott et al., 1989; Dunn et al., 1995; Menegola et al., 2005; Zimmer et al., 2012). Indeed, the H9 cells showed inhibition of calcification at lower concentrations than the RIV9 cells (Fig. 4.4A). In addition, this differentiation defect occurred at concentrations lower than those that caused cytotoxicity (Fig.4.4B, C). Correlating cytotoxicity with differentiation inhibition of the respective compounds revealed lower R² values in the H9
cells (Fig. 4.4D), again suggesting that H9 cells were more sensitive to neural crest inhibitory chemicals than RIV9 cells.

Figure 4.4. Concentration-response curves for representative neural crest-associated inhibitory compounds. (A-C) Human embryonic stem cells, induced pluripotent stem cells, and foreskin fibroblasts were analyzed on day 20 for concentration dependent effects on differentiation (Ca²⁺) and cell viability (mitochondrial dehydrogenase activity) after exposure to chemicals known to interfere with neural crest development in vivo, n=5±SD. *p<0.05 One-Way ANOVA exposed H9 hESCs versus solvent only. ΔP<0.05 One-Way ANOVA exposed RIV9 hiPSCs versus solvent only. §P<0.05 Student’s t-test H9 hESCs versus RIV9 hiPSCs. (D) Linear regression analysis between the endpoints of cell viability and differentiation (calcium) for neural crest inhibitory compounds. hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; MAA, methoxyacetic acid; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide.
Shortening assay duration by determining TWIST1 expression

Previous studies by our group and others have found positive associations with the measurement of lineage gene expression and prediction of embryotoxic potential (Schulpen et al., 2013, 2015b; Suzuki et al., 2011; Theunissen et al., 2012; zur Nieden et al., 2001, 2004). In addition to providing an objectively quantifiable endpoint, these means of differentiation analyses have also often led to the shortening of assay duration. Specifically, this is achieved by determining the expression of genes found in progenitors rather than fully differentiated cells. We thus hypothesized that the ability of the test compound to inhibit calcification may also correlate to a defect in specific mRNA expression patterns. Specifically, we aimed to test whether the determination of TWIST1 levels, expression of which occurs early during differentiation prior to mineralization (Sparks et al., 2018), would shorten the chemical assessment and equally predict the chemical’s in vivo embryotoxicity classification. This hypothesis was based on prior literature that has described TWIST1 as an epithelial to mesenchymal transition marker with an active role in mediating osteogenic potential, specifically during the specification of cranial bone (Yen et al., 2010). Prior research has suggested that TWIST1 promoter methylation status and expression patterns can be indicative of osteogenic yield in pluripotent stem cell cultures (Sparks et al., 2018).

The assessment of TWIST1 mRNA expression produced ID50 values lower than those seen for calcification. With H9 cells, ID50TWIST1 was lower for three of the eight tested chemicals (13cisRA, MAA, triademenol). RIV9 cells had six out of the eight chemicals tested with decreased TWIST1 (Table 4.1; Appendix Figure 1.4.3). Due to the
higher sensitivity of the TWIST1 mRNA endpoint over the calcium assay in most of the chemicals, the correlation between these two endpoints was lesser for the RIV9 cells than for the H9 cells (R² 0.34 versus 0.84) (Fig. 4.5A). Notably, the biostatistical model classified 13cisRA as strong embryotoxicant (class III) in the endpoint of TWIST1 gene expression for both cell lines (Table 4.1).

Figure 4.5. Half-maximal inhibitory doses for cytotoxicity (IC₅₀) as determined from concentration-response curves with MTT assay and differentiation (ID₅₀) with Arsenazo calcium assay and quantitative PCR for TWIST1. (A) Linear regression analysis showing degree of correlation between different endpoints. (B) Bar graphs charting the established half-maximal inhibitory doses for all PSC endpoints. *p<0.05 Student’s t-test ID50 versus IC50, †p<0.05 Student’s t-test H9 hESCs versus RIV9 hiPSCs. 5FU, 5-fluorouracil; 13cisRA, 13-cis Retinoic Acid; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; IC50, half-maximal inhibitory concentration of cytotoxicity; ID50, half-maximal differentiation inhibiting concentration; MAA, methoxyacetic acid; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PenG, penicillin G.
Integration of concentration-response curves and biostatistical models to predict skeletal developmental toxicity

Developmental toxicity of a compound can be assessed by its cytotoxicity or ability to inhibit differentiation. In our model, we specifically aim to uncover the effects of the toxicants on the developing skeleton specifically instead of widespread cytotoxicity. Calcification and TWIST1 mRNA endpoints showed the same embryotoxicity classification of PenG, 5FU and atRA (Table 4.1) for both hPSC lines. However, the comparison between IC50 and ID50 suggested that some compounds were cytotoxic while others had the ability to inhibit differentiation prior to cytotoxic induction. (Fig. 4.5B). Determined by the ID50Ca2+, 5FU, atRA, and 13cisRA were cytotoxic to H9 cells while the NC chemicals only inhibited differentiation. 5FU, atRA, and cyclopamine were cytotoxic to H9 cells as assessed by ID50TWIST1. ID50Ca2+ of the RIV9 cells showed 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 Cytotoxicity (MTT)</th>
<th>ID50 Differentiation Inhibition (Ca2+)</th>
<th>class</th>
<th>IC50 Cytotoxicity (MTT)</th>
<th>ID50 Differentiation Inhibition (TWIST1)</th>
<th>class</th>
</tr>
</thead>
<tbody>
<tr>
<td>PenG (class I)</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>I</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>I</td>
</tr>
<tr>
<td>5FU (class III)</td>
<td>0.090 ± 0.019</td>
<td>0.003 ± 0.002</td>
<td>III</td>
<td>0.098 ± 0.014</td>
<td>III</td>
<td>0.000158 ± 0.00002</td>
</tr>
<tr>
<td>atRA (class III)</td>
<td>0.091 ± 0.039</td>
<td>0.1 ± 0.020</td>
<td>III</td>
<td>0.146 ± 0.0048</td>
<td>III</td>
<td>0.0069 ± 0.0029</td>
</tr>
<tr>
<td>13cisRA</td>
<td>1.047 ± 0.127</td>
<td>1.97 ± 0.658</td>
<td>III</td>
<td>0.0911 ± 0.0005</td>
<td>III</td>
<td>0.643 ± 0.31</td>
</tr>
<tr>
<td>5FU, atRA, and 13cisRA</td>
<td>10.02 ± 2.04</td>
<td>8.5 ± 2.0</td>
<td>I</td>
<td>30.04 ± 43.18</td>
<td>I</td>
<td>160 ± 31.07</td>
</tr>
<tr>
<td>Methoxycetic acid</td>
<td>100 ± 9.5</td>
<td>23 ± 1.3</td>
<td>I</td>
<td>0.0043 ± 0.005</td>
<td>II</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Triadenasin</td>
<td>13 ± 1.4</td>
<td>0.32 ± 0.05</td>
<td>II</td>
<td>0.0333 ± 0.02</td>
<td>II</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Triadenenol</td>
<td>11 ± 1.2</td>
<td>1.8 ± 0.1</td>
<td>II</td>
<td>0.0171 ± 0.02</td>
<td>II</td>
<td>12 ± 6.5</td>
</tr>
</tbody>
</table>

| Table 4.1. Half-maximal inhibitory concentrations for all tested endpoints and prediction of embryotoxicity classes.
out of the 8 tested compounds were cytotoxic. All compounds solely inhibited differentiation in the RIV9 ID50TWIST1 screen.

Discussion

Over the past decade multiple efforts have been launched to develop human-based in vitro assays to identify and predict in vivo toxicity to determine the safest chemicals. Initial studies examined various techniques to quantify cell viability (Adler et al., 2008a, b; Pal et al., 2011). This was followed by different methods to analyze the differentiation behavior of the cells (Colleoni et al., 2011; Krug et al., 2013), often for compound exposure on fully differentiated cells (Jahnke et al., 2013; Mathur et al., 2015). While the latter studies have their own merit, compound exposure of differentiated cells represents toxicity only and not developmental toxicity, thus are inadequate to understand the potential of a compound to induce birth defects. In addition, most of these studies were lacking a comparison between mouse and human cells to evaluate the predictivity of the in vitro models. Indeed, comparisons of global gene expression changes in differentiating human and mouse ESC-derived neurons during valproic acid exposure, suggested a substantial difference in response between the mouse and the human cells (Schulpen et al., 2015a, b). While only 988 genes were commonly regulated, a total of 4913 genes were differentially regulated between the two species, likely leading to a differential exposure outcome between the species.

Although representing key steps towards developing a human-based assay, prior studies with human-derived ESCs did not incorporate all three endpoints of the original
mouse EST (mEST). In addition to the assessment of cell viability and differentiation inhibition on ESCs, the mouse EST compares these two endpoints to the cell viability of 3T3 fibroblast cells of the same test compound (Scholz et al., 1999), signifying the cytotoxic effect of the test chemical on the mother. Together, the three endpoints are summarized in a biostatistical model that allows for the classification of the test compound into three embryotoxicity classes: non-, weak and strong embryotoxic (Genschow et al., 2000, 2002). To this end, we tested a series of chemicals in our human pluripotent stem cell (hPSC)-based developmental toxicity assay to accurately determine compound potency using all three original EST endpoints. Furthermore, we sought to demonstrate species differences upon compound exposure. Lastly, we determined whether our test was sensitive enough to distinguish between compound inhibition of different embryonic lineages.

Human ESCs display multiple advantages in the field of toxicology testing due to their pluripotency, which includes unlimited proliferation capability and the ability to generate cells of all three germ layers from a human source. While mouse and human ESCs can both differentiate into the same cell types, there are differences between the ESCs that may inhibit the ability to extrapolate rodent data for human predictions. For example, undifferentiated human ESCs form flat colonies and are seeded as small cell aggregates, while mouse ESCs can be dissociated as single cells and form 3D colonies. Similarly, murine ESCs require the presence of leukemia inhibitory factory (LIF) for maintenance of a pluripotent state (Nichols et al., 1990), which is insufficient to maintain pluripotency in human ESCs (Humphrey et al., 2004). There is also variability of in vitro differentiation
into cardiomyocytes. Cardiac differentiation is induced via embryoid bodies, which are either formed using the hanging drop protocol (mouse ESCs) or by culturing cells in suspension (human ESCs). Also, the timing with which contractile activity is first detected and for how long it is sustained differs between cells of the two species (Kehat et al., 2001; Metzger et al., 1994; Wobus et al., 1991). Thirdly, the efficiency with which contractile structures are formed varies substantially (Chow et al., 2013; Kehat et al., 2001).

Based on such differences, we hypothesized that including human cell lines can improve the ability of the EST to detect embryotoxicants with relevance for human exposure. Our results indeed demonstrated the benefit of including the developmental toxicity of hESC and hiPSC-derived osteoblasts in comparison to the osteogenic endpoint of the mESC based EST (mESTo). Contrasting cell viability and calcification of exposed mouse ESCs, hESCs, and hiPSCs with inhibition of cell viability in somatic fibroblasts equally predicted the classification of 5FU, arRA, and PenG according to their known in vivo teratogenicity classes. To our knowledge, this is the first time that all three of the mouse EST endpoints were tested together in a human model. However, despite predicting the same developmental toxicity classes as mouse cells, the IC50 and ID50Ca for 5FU was developmental toxic to human ESCs at a concentration 100-fold lower than mouse cells. This may indicate that the human PSC-ESTo is a potentially more sensitive in vitro embryotoxicity screening assay to determine the adverse effects of a test compound on the developing skeleton than the mouse ESC-based assay. As such, the human-based test could yield more appropriate results to set human exposure limits during risk assessment and subsequent legal regulation of chemicals.
In vitro differentiation of hESCs into osteoblasts is a robust process and does not require specially trained personnel to detect the presence of black deposits in culture signifying osteoblast mineralization (zur Nieden et al., 2003). Indirectly, osteoblastogenesis can be quantitated through calcium content and a differentiation endpoint (zur Nieden et al., 2007). The differentiation of osteoblasts as determined by the level of calcium is a suitable endpoint for the EST to measure the osteotoxic potential of compounds (zur Nieden et al., 2010a,b; Walker et al., 2015). However, calcium content alone cannot detect the potency of a compound and could cause false negative results. This is possibly due to the number of proliferative cells in culture that cannot be controlled and will vary between all treatments, including the control. Previous studies have observed the robustness of calcium content as an endpoint for osteoblast differentiation in the mouse EST (de Jong et al., 2012; Chen et al., 2015). In these studies, however, there are discrepancies in calcium levels for the tested toxic compounds. Specifically, Chen et al. reported increased levels of calcium in 5FU treated cells. Chen et al. (2015) and de Jong et al. (2012), both concluded that measuring the amount of calcium was not sensitive for the osteoblast EST. Instead, our prior work suggested the detection of calcium ions in the extracellular matrix to be a suitable endpoint for detecting a chemical’s potential to interfere with osteogenesis when normalized to the overall protein content (zur Nieden et al., 2010a, b; Kuske et al., 2012). Here, we apply this endpoint to the human ESTo since it is an indicator of the efficiency of the differentiation protocol. A potential reason for the differential conclusion between these studies may have been the failed accounting for the number of viable cells in culture in the other studies.
In human PSCs, 13cisRA potently inhibited osteogenesis in both cell lines. We specifically chose to test 13cisRA because it is highly teratogenic to humans resulting in cardiovascular, central nervous system, limb, and craniofacial abnormalities. It has been well documented that humans are more sensitive to the developmental toxic effects of 13cisRA than rabbits and rodents (Kochhar et al., 1984). To elicit similar developmental toxic effects in rodents, doses of 13cisRA can be up to 100-fold higher than what is harmful in humans (Kraft et al., 1987; Tzimas et al., 1994; Nau, 2001). Potentially, such species variability may be attributed to different metabolism and pharmacokinetics (Nau, 1986; 1995; 2001). Indeed, our human cell-based *in vitro* study supported the greater sensitivity of human cells to 13cisRA and classified 13cisRA as weakly toxic, while the murine embryonic stem cells classified it as nontoxic.

Overall, the development of an *in vitro* assay for skeletal toxicity is aggravated by the complexity of the bone formation process, including different embryonic origins of the mesenchymal cells that turn into osteoblasts and the process by which this occurs. Depending on whether neural crest cells or mesodermal progenitors are impacted, craniofacial or limb defects will ensue. For instance, cyclopamine, methoxyacetic acid, and the triazoles triadimefon and triadimenol have known effects on neural crest cells and thus the craniofacial skeleton (Scott et al., 1989; Dunn et al., 1995; Menegola et al., 2005; Papis et al., 2006; Di Renzo et al., 2007; Zimmer et al., 2012). We tested the ability of our hPSC-ESTo to identify these compounds as inhibitors of osteogenesis. Our focus was to select compounds that would induce craniofacial abnormalities to the developing fetus and determine if our two hPSC lines can yield differential sensitivity to the compounds. Our
previous study has uncovered that H9 hESCs predominantly generate osteoblasts of the NC lineage and our RIV9 hiPSCs make mesoderm-derived osteoblasts (Sparks et al., 2018). In early development, when the craniofacial region is derived from neural crest cells as they migrate from the neural tube, sonic Hedgehog (Shh) pathways are necessary for neural crest formation and proper facial development (Jeong et al., 2004). Cyclopamine, as a Shh inhibitor has been known to induce limb and craniofacial teratogenesis in variety of species and targets neural crest cells specifically, while other cell types such as trunk neural crest cells are resistant to its effects (Dunn et al., 1995). Methoxyacetic acid (MAA) is a general growth retardant with a relatively long half-life when injected into pregnant non-human primates (Scott, 1989). MAA treated rats have shown to elicit skeletal malformations (Brown, 1984). Other studies demonstrate embryonic effects on the suture of the craniofacial region (Teixidó, 2012). Traidimefon and triadimenol are widely used as antifungal agents in agriculture and are derived from triazoles. Vertebrate systems from xenopus to zebrafish to rodents have resulted in craniofacial malformations due to toxicant exposure (Mengola et al., 2005; Papis et al., 2006; Grimes et al., 2008). Triadimenol and triadimefon have been shown to alter neural crest migration in human models and have well documented evidence that both chemicals alter neural cell migration in vivo and in vitro (Zimmer, 2012). Here, the H9-ESTo showed a dose-dependent inhibition of osteogenesis to these compounds, while they did not inhibit RIV9 calcification and showed cellular toxicity only in the highest dose of Triadimefon. Since our previous study presented in chapter 2 has uncovered that H9 hESCs predominantly generate osteoblasts of the neural crest lineage and that the RIV9 hiPSCs predominantly made mesoderm-derived
osteoblasts (Sparks et al., 2018), the higher potency of these chemicals in the H9-ESTo suggests the expected potential targeting of NC cells by these compounds. Thus, the concentration-response curves seem to indicate the potential of our test cell lines to distinguish osteotoxicity as it is chemical-specifically induced in the different osteogenic lineages.

We also evaluated the effects of the compounds at the molecular level based on the expression of an early mesenchymal gene, \textit{TWIST1}, with the goal to reduce the test duration. When selecting a candidate gene for assessment in the EST, it is necessary to choose markers exclusive to the lineage under investigation and not such that are ubiquitously expressed in other tissues. This is due to the pluripotent nature of the cells, which, even when triggered into a certain lineage, will generate contaminating cell types unless some purification step is included. As such, we have previously assessed osteogenic differentiation inhibition with murine ESCs based on the expression of \textit{osteocalcin} (\textit{OCN}) (zur Nieden et al., 2004). \textit{OCN} mRNA and protein expression is exclusively associated with a maturation stage of osteoblasts, at which extracellular matrix is secreted that is then calcified (Aubin and Liu, 1996). Therefore, \textit{OCN} expression can successfully be detected at later stages of ESC osteogenic differentiation (Sparks et al., 2018; zur Nieden et al., 2003). In the current study, \textit{TWIST1} was selected over \textit{OCN} as it is expressed during early stages of osteogenesis and is known to have an important role in proper bone development (Miraoui et al., 2010). In mesenchymal stem cells, which are more specialized than ESCs, it is the down-regulation of this gene that directs osteoblast differentiation (Miraoui et al., 2010), while continued upregulation of \textit{TWIST1} will result in the maintenance of a stem
cell state (Goodnough et al., 2012). Conversely, the absence of TWIST1 in vivo leads to Saethre-Chotzen syndrome, which is characterized by untimely closure of the cranial sutures (Huang et al., 2014; Isenmann et al., 2009). Our results using TWIST1 mRNA analysis indicate that the compound’s innate toxicities can be assayed correctly when examining levels of this mRNA in hESCs. However, TWIST1 was not a suitable endpoint for the RIV9 hiPSCs. TWIST1 mRNA was dose dependently downregulated in the hiPSCs exposed to the NC chemicals. This expression data does not correlate to the calcification data and potentially resulting in a false-positive evaluation. While seemingly suitable for the hESCs, this finding warrants further studies but overall shows the need to include molecular endpoints in the EST that are cell-type specific. Previously assessed mRNA levels of Collagen type I or Sparc show the need to find early lineage markers that are tissue-specific (de Jong et al., 2012). While treatment of murine ESCs with methotrexate (MTX), a childhood chemotherapy agent, resulted in the downregulation of Col1A1, this down-regulation may not solely reflect an inhibitory effect on osteogenic differentiation, since Col1A1 is expressed in virtually every tissue including lungs, liver, kidney and muscle (Lejard et al., 2011; Mia et al., 2014; Tsuchida et al., 2003; Uezumi et al., 2011). Similarly, the potency of MTX to reduce Col1A1 expression was observed at a concentration analogous to its IC<sub>50</sub>, suggesting it was either inhibitory to Col1A1 expression due to the general cytotoxicity of the compound or that the assessed time point did not reflect the actual differentiation stage of the cells. Indeed, Col1A1 expression is typically found in pre-osteoblasts (Ducy, 2000) possibly explaining why other studies find a down-regulation of this gene beyond a certain differentiation stage (Bilousova et al.,...
2011; Darcic et al., 2001; Kalajzic et al., 2002; zur Nieden et al., 2003). According to the in vivo teratogenicity of MTX, which is known for skeletal defects due to arrest of bone growth, reducing proliferation of chondrocytes, and inducing apoptosis in chondrocytes (Fan et al., 2009; Nilsson et al., 1984; Xian et al., 2008, 2007), treatment of murine ESCs with this compound did also down-regulate Runx2, a master regulator of osteogenesis and exclusive to that lineage (Ducy et al, 1997). While seemingly a predictive indicator of skeletogenic teratogenicity, Runx2 is expressed later during osteogenesis than TWIST1 (Yousfi et al., 2002) and we therefore chose TWIST1 for this study. All data together suggests no one gene is a suitable catch all for assessing developmental toxicity across multiple cell lines and species, and a combination of markers would be the best indicator of embryotoxic potential.

Another alternative assessment in the field of embryotoxicity are high throughput screens (HTS), that may potentially meet the demand of testing the thousands of compounds entering the environment each year. Using a reporter gene assay, van de Burg et al. (2015) took a mechanistic approach in creating a predictive, 82%, high throughput assay to assess reproductive toxicity. Though the study tested for one tissue endpoint and lacked the recapitulation of developing cells that hESCs can provide, the system demonstrates the effectiveness and potential of such systems. Kameoka et al. (2014) performed a fast and predictive teratogenicity screen on hESCs differentiated into tissues of the three germ layers. This testing strategy demonstrates how a developmental toxicant can perturb an essential process, gastrulation, which could lead to a birth defect. While predictive, the study was limited in demonstrating inhibition of a functional tissue, resulting
in unknown anomalies. Additively, adverse outcome resulting from exposure during later development was missed. In the hESTo, however, cells are exposed throughout all phases of development and this assay thus shows the capability to uncover developmental osteogenic toxicity.

**Conclusion**

We have implemented the use of human pluripotent stem cells to improve the ability of the EST for human risk assessment of compounds. The assay has shown sensitivity of osteogenically differentiating cells going through different developmental pathways in test compounds. The human cells successfully predicted the embryotoxic potential of 5FU, 13cisRA, and 13cisRA with contrasting sensitivity compared to the mouse ESCs. This emphasizes interspecies differences in susceptibility to environmental toxicants. The hESC-derived osteoblasts showed sensitivity to chemicals known to affects NC cells while the hiPSCs showed no inhibition of osteogenesis, noting that researchers should fully understand the differentiation process of the cells being used. In sum, the present study provides evidence that the use of human pluripotent stem cells may enhance the EST and proposes that the human EST is a reliable indicator for testing human hazards.
References


CHAPTER 5

Tobacco-induced oxidative stress disrupts osteogenic differentiation in human embryonic stem cells.

Nicole RL Sparks and Nicole I zur Nieden

Introduction

Every 4.5 minutes, a baby is born with a birth defect in the United States. According to the Center for Disease Control, birth defects are the major cause of infant deaths, accounting for 1 in 5 infant deaths. Of the babies born with an abnormality, approximately one-third have a defect of the head and face (Trainor, 2010) including cleft lip/palate, improperly fused skull, small or absent skull and facial bones, and malformed nose, eyes, ears, and teeth (Carmichael et al., 2008; MOD, 2010). Both functionally and socially such birth defects can have a significant impact on the patient's quality of life. For example, the CDC estimates the lifetime therapy costs for children born each year with cleft lip and/or palate to be US$697 million (Trainor, 2010). Causes of birth defects can be attributed to the environment, pharmaceuticals, genetics, viruses, nutrition, and diet (NCBDDD, CDC). Approximately 7% of all congenital defects are caused by exposure to known teratogens (Chung, 2004), which is defined as any substance (i.e., chemical, infectious agent, or physical condition) that has the ability to cause abnormal development to the embryo or fetus while in the mother’s womb (Polifka and Friedman, 2002). It is specifically during
periods of rapid cell division and differentiation that the embryo is most susceptible to teratogenic agents (Chung, 2004). Despite the great strides made in developmental biology in the last century, the adverse mechanisms associated with embryonic exposure to teratogens is poorly understood and, as such, is of great interest.

Tobacco products are one of the most common environmental toxicants/teratogens and tobacco exposure is the single most preventable cause of disease, disability, and death in the United States (CDC). Through anti-smoking initiatives, the prevalence of smoking-related diseases has declined since 1965, from 43% to 18% today (Hall and Doran, 2016). However, present tobacco use is attributed to nearly 500,000 deaths per year in the United states, including an estimated 42,000 deaths from secondhand smoke exposure, and 8.6 million people live with a serious illness caused by smoking (CDC; Hall and Doran, 2016). The costs associated to smoking-related illnesses in the United States is estimated to be more than $300 billion per year (Xu et al., 2014; U.S. Department of Health and Human Services, 2014a). Adverse effects of tobacco smoke have been demonstrated by epidemiological and in vivo studies that associate smoke with diseases affecting the heart, lungs, and various cancers (WHO, 2011). In addition, disorders of the adult skeleton have been linked to cigarette smoke. Tobacco use has been associated with increased risk of developing osteoporosis, increased risk of fractures, delayed rate of bone healing, 4-5% decreased bone mineralization, and obstruction to bone surgery results (Akhter et al., 2005; Rothem et al., 2009; AAOS, 2016).

Maternal smoking during pregnancy exposes the unborn fetus to tobacco constituents. Of the 7000 constituents of cigarette smoke, many chemicals like nicotine and
carcinogenic compounds can cross the placenta (Wickström, 2007; Bruin et al., 2010). Of all the children today, it is estimated that almost 1 out of 13 will die prematurely due to smoking (AAOS, 2016). Furthermore, in infants and children, secondhand smoke is the number one cause of asthma attacks, respiratory and ear infections, and sudden infant death syndrome (U.S. Department of Health and Human Services, 2006; 2014b). Despite this knowledge, 10-20% of United States women smoke during pregnancy with the highest smoking rates attributed to women of lesser education and low socioeconomic background (Tong et al., 2009; Curtin and Matthews, 2016). Exposure to tobacco smoke increases the mother’s risk of spontaneous abortion, premature delivery, infertility, and fetal abnormalities such as long-term effects on brain function and cognition and improper development of the skeleton (Shiverick and Salafia, 1999). Furthermore, maternal smoking has shown to increase the chance of cleft lip and/or palate and premature closure of coronal sutures (craniosynostosis) (Carmichael et al., 2008; MOD, 2010). While studies have demonstrated the detrimental effects of maternal smoking to the developing skeleton, there is a lack of investigation to understand the pathogenic mechanisms of tobacco-induced skeletal teratogenicity in the developing embryo.

The known adverse health outcomes associated with tobacco use has led to the public misconception that alternative forms of tobacco like ultra-filtered and low nicotine/tar cigarettes or non-combustible chewing tobacco are safer than traditional cigarettes (Byron et al., 2017). Pregnant women who experience difficulty with smoking cessation may use these perceived safer alternatives, termed harm-reduction tobacco products (HRTPs). Maternal use of low nicotine and tar cigarettes, like Camel Blue, is a
risk factor for low birth weight (Mitchell et al., 2002). In comparison to cigarette smoking, it has been suggested that Snus can be a less harmful option due to not forming the combustible constituents of smoke (Ashford, 2016). However, there is growing evidence out of Sweden that maternal Snus use may be associated to oral cleft birth defects (Gunnerbeck et al., 2014). While tobacco use during pregnancy has been linked to cognitive detriments, low birth weight, and skeletal defects, there is a lack of studies focusing on how tobacco disrupts bone development. With the increased use of HRTPs, there is a gap in knowledge of the molecular mechanisms of HRTP-induced skeletal teratogenicity.

**Prior data**

I have previously established a human ESC in vitro model of osteogenesis (chapter 2) and adapted the protocol to be used in a human embryonic stem cell test to predict the osteotoxicity caused by chemical compounds (chapter 4). Our lab has used the differentiation protocol and human EST to test popular tobacco brands for their ability to inhibit osteogenesis in differentiating hESCs. Since tobacco smoke exposure can occur through mainstream (MS) smoke, which is actively inhaled by the smoker, and sidestream (SS) smoke, which burns off the tip of the cigarettes, both forms of smoke were tested. In addition, exposure can also happen orally using non-combustible products such as chewing tobacco, also called Snus. Among the tested tobacco products were Camel (conventional) MS and SS smoke, Camel Blue (previously known as Camel Lights) MS and SS smoke, and Camel Suns tobacco extract (STE). Conventional Camel and Camel Blue MS smoke
showed no cytotoxicity nor detrimental effects to osteogenesis (Fig 5.1A). In contrast, the SS smoke of conventional Camel and Camel Blue and Camel STE treated cultures, dose-dependently inhibited osteogenesis. Of importance, the differentiating hESCs exposed to Camel Blue SS and STE showed a differentiation defect at concentrations that did not elicit cytotoxicity, whereas the conventional Camel SS caused inhibition due to cytotoxicity (orange arrows).

In zebrafish, developmental exposure to Camel, Camel Blue, and STE induces cartilaginous defects in the developing head and spine as well as a mineralization delay (Fig. 5.1C). In mice that were exposed to Camel Blue and STE in utero, multiple skeletal defects can be found at E17.5 that resemble metabolic bone disorders and are characterized by low bone mineral density (Fig 5.1D). In sum, using in vitro human pluripotent stem cells (hPSCs) and in vivo mouse embryo models, we provide here the first evidence that HRTPs are toxic to the developing skeleton. This chapter will investigate the molecular mechanisms behind the observed differentiation defect. The data revealed that effective doses of Camel, Camel Blue, and STE generated excessive levels of the reactive oxygen species (ROS), superoxide anion and hydrogen peroxide, during early stages of osteogenesis along with elevated NADPH oxidase activity. However, HRTPs generated ROS at intermediate levels compared to conventional Camel SS, suggesting a mechanism for the difference between cytotoxicity caused by the conventional tobacco and the subtoxic differentiation inhibition noted in HRTP-treated cells. Together these data support the notion that HRTPs initiate oxidative stress and disrupt embryonic skeletal development as will be outlined below.
Figure 5.1. The embryotoxicity associated with tobacco exposure. A) Concentration-response curves of Camel, Camel Blue, and Camel Snus Tobacco Extract (STE). Concentration-responses were determined by MTT assay of H9 hESCs and HFF cells and calcium assay of H9 hESCs; n=5, *p<0.05 One-Way ANOVA and Tukey HSD exposed versus untreated control. Data courtesy of Ivann Martinez, zur Nieden lab. B) Compiled list of IC50 and ID50 values of all screened tobacco products. Non-effective and effective doses were determined by the curves. C) Zebrafish exposure to Camel or STE negatively regulates pharyngeal arch development. On day 5 post fertilization, a COL2-reporter, which is expressed early in development, distinguished variable craniofacial defects between exposure to the two tobacco products. White arrows point to shortened development of the head and absent arches. Exposure also caused curvature of the spine (black arrows). Data courtesy of Dr. Maria Bondesson-Boulin and Omran Karmach, zur Nieden lab. D) Mouse pups exposed in utero to Camel Blue or STE exhibited porous skulls (white arrow heads). hESCs, human embryonic stem cells; HFF, human foreskin fibroblasts; IC50, half-maximal inhibitory concentration of cytotoxicity; ID50, half-maximal differentiation inhibiting concentration; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide. Col2:mccherry labels chondrocytes expressing collagen 2.
Materials and Methods

hESC culture

Human ESCs of the H9 line were maintained on Matrigel (BD Biosciences) coated plates in serum-free mTeSR® medium (Stem Cell Technologies). Cultures were passaged every 4 days with accutase.

Osteogenic differentiation

Human ESC confluent colonies were given control differentiation medium consisting of Dulbecco’s modified Eagle's medium (DMEM; Gibco, US) containing 15% FBS (Atlanta Biologicals, US, selected batches), 1% non-essential amino acids (NEAA; Gibco, US), 1:200 penicillin/streptomycin (Gibco, US), and 0.1 mM β-mercaptoethanol (Sigma, US) for 5 days as described (Sparks et al., 2018; Chapter 2). Subsequently, control differentiation medium was supplemented with osteogenic factors: 0.1 mM β-glycerophosphate (βGP; Sigma, US), 50 µg/ml ascorbic acid (AA; Sigma, China), and 1.2x10^{-7} M 1,25(OH)_{2} Vitamin D_{3} (VD_{3}; Cayman Chemical, US).

Production of tobacco solutions

Commercially available conventional (Camel) and harm reduction (Camel Blue) brands of cigarettes were purchased from a local retail dealer and used to make mainstream (MS) and sidestream (SS) smoke solutions with a method described previously in detail (Knoll and Talbot, 1998). Smoke solutions were generated using a University of Kentucky smoking machine that took a 2.2 second puff of MS every minute. MS smoke solution was
generated by pulling 30 puffs of MS smoke through 10 ml of DMEM culture medium. During MS smoke production, SS smoke solution was produced by collecting the smoke that burned off the end of the cigarette and pulling it through 10 ml of DMEM. SS smoke was collected continuously, while MS smoke was collected during each puff. Both MS and SS solutions were made at concentrations of 3 puff equivalents (PE). One PE of MS smoke is the amount of smoke in one puff that dissolves in 1 ml of culture medium. For SS smoke, one PE is the amount of SS smoke that dissolves in 1 ml of culture medium during one minute of burning. Immediately after preparation, smoke solutions were filtered through a 0.2 µm Acrodisc® PSF Syringe Filter (Pall Corporation, US), aliquoted into sterile Eppendorf tubes, and stored in a -80°C freezer until used. Desired PEs were acquired through serial dilutions, and experiments were done using either MS or SS at concentrations as indicated and an untreated control.

Tobacco extract was created at 10% (w/v) in DMEM. Resulting solution was centrifuged for 10 min at 450 × g, to remove large debris. Supernatant was collected and centrifuged at 13,000 × g for 1 h, to remove finer debris. pH was adjusted to 7.4 and extract sterilized through a 0.45µm filter. FBS was added to a final concentration of 15%.

Antioxidant treatment

The following were added at the start of osteogenic differentiation and tobacco treatment (previously described) at a final concentration of: ascorbic acid (AA; Sigma-Aldrich, USA) 100 µM, 10 µM dl-α-tocopherol acetate (vitamin E; Supelco, Sigma-
Aldrich, USA), and glutathione reduced ethyl ester (GSH-EE; Sigma-Aldrich, USA) 500 μM. The antioxidant medium was replaced with each media change.

RNA isolation and quantitative PCR

Total RNA was extracted with the NucleoSpin RNA II protocol (Macherey Nagel). RNA concentration was determined with a NanoDrop® 1000 spectrophotometer (Thermo Scientific) at 260 nm. 25 ng of total RNA was used for cDNA synthesis with a mastermix including 1X reaction buffer, 0.5 mM dNTPs, 20 U/μL RNase inhibitor, 0.8 U/μL reverse transcriptase, and 1 μM random primer. 25ng cDNA transcripts were used for quantitative polymerase chain reaction (qPCR) with SYBR green on the CFX96 cycler (BIO-RAD). Reactions were setup for 10 minutes of denaturing at 94°C, followed by 40 cycles of denaturing at 94°C, and annealing/elongation at 60°C for 45 seconds. The n-fold expression in target samples was calculated with the ΔΔC₀ method by standardizing C₀ values to GAPDH expression. Primer sequences used in this study can be found in Table 5.1.

Superoxide anion (O₂⁻) detection

Generation of superoxide anion was determined using a Lumimax Superoxide Anion Detection Kit (Agilent Technologies, USA). H9 cultures were trypsinized, washed with PBS, and resuspended in 1mL of fresh medium to incubate for 30 minutes at 37°C. A total of 5 × 10⁵ of the cells were resuspended in 190 μL of superoxide anion (SOA) assay medium and 5 μL of 4.0 mM luminol solution plus 5 μL of 5.0 mM enhancer medium were
added to the SOA medium and incubated at room temperature for 30 min. The chemiluminescent light emissions of superoxide anion were measured with a luminometer (Lucetta™).

_NADPH Oxidase O_2^- detection_

NADPH oxidases (NOX) are superoxide anion generating enzymes. Superoxide anion, O_2^-, was detected by the chemiluminescent probe lucigenin, where lucigenin is reduced by O_2^- to a lucigenin cation radical (Wang et al., 2011; Shah et al., 2013). Briefly, cultured cells were washed with 1X PBS and lysed in 300 µL of lysis buffer (20 mM KH2PO4, pH 7.0, 1 mM EGTA, protease inhibitors). 100 µL of lysed cells were added to 900 µL of assay buffer consisting of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin (Sigma M8010), and 100 µM NADPH (Sigma N5130). Chemiluminescent light emission in terms of relative light units was measured in a luminometer every 3 min for 30 min. There was no measurable activity in the absence of NADPH. Protein content was measured with the Lowry method using the Bio-Rad DC protein assay reagent. All measurements were normalized to its respective protein amount. Superoxide anion production was expressed as relative chemiluminescence (light) units (RLU)/mg protein.

_MitoSOX™ mitochondrial (O_2^-) detection_

Mitochondrial superoxide formation was detected by incubating cells in the dark with 2.5 µM MitoSOX™ Red dye (excitation/emission at λ = 510 nm/580 nm;
ThermoFisher M36008) in PBS for 10 min. The average fluorescence intensity per cell for each experimental group of cells was calculated using NIH ImageJ analysis software as described by Fitzpatrick (2014) and Jensen (2013).

Detection of hydrogen peroxide

Dihydrorhodamine 123 (Invitrogen D23806) is an uncharged and nonfluorescent H$_2$O$_2$ indicator that can passively diffuse across membranes where it is oxidized to cationic rhodamine 123 which localizes in the mitochondria. Cells were incubated in 1µM of DHR-123 in PBS for 30 min at 37°C. Cells were washed twice with PBS and harvested in 500µL cold PBS for flow analysis in Beckman Coulter Flow cytometer detected at excitation/emission at $\lambda = 485/535$ nm. Gating was set using unstained control samples by forward scatter and side scatter light. For each sample, 10,000 events were collected. Increased green fluorescence intensity indicates increased ROS H$_2$O$_2$.

Mitochondrial Membrane Potential ($\Delta\Psi_m$)

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazoyl carbocyanine iodide; Invitrogen, T3168) is a lipophilic cationic dye that enters the inner mitochondrial matrix in its monomeric form when the mitochondrial membrane is polarized. When the mitochondrion has a high $\Delta\Psi_m$, the dye crosses the membrane and forms J-aggregates, which appear red under UV light. If $\Delta\Psi_m$ is low, the dye remains in its monomeric form and fluoresces green. The membrane-sensitive dye was prepared at 1 µg/mL working solution in PBS from stock solution (5mg/mL in DMSO) prior to use. Cells were harvested
in microcentrifuge tubes, centrifuged, and incubated with 500µL of working solution for 15 min in a humidified incubator at 37°C, 5% CO₂. Following staining, the cells were washed twice in PBS and resuspended in cold PBS for flow analysis in Beckman Coulter Flow cytometer. To exclude debris, samples were gated to unstained samples from the same differentiation day based on forward scatter and side scatter light. Using the “medium” setting flow rate, 10,000 events were collected. Mitochondrial potential was detected at excitation/emission at λ = 590/610 nm for JC-1 aggregates and excitation/emission at λ = 485/535 nm for monomers.

**Superoxide dismutase (SOD)**

Cultures were washed with PBS, harvested in 300 µL of RIPA buffer, and stored at -20 °C. Once thawed, the SOD assay kit was utilized per manufacturer’s instructions (Cayman Chemical 706002). 10 µL of samples were combined with 200 µL of radical detector. The addition of 20 µL xanthine oxidase initiated the reaction, followed by a 20-minute room temperature incubation. The absorbance was measured at 450 nm. Absorbance values for each sample were compared to a 7-point SOD standard curve (provided by the kit) to determine SOD activity. The SOD activity of each sample was normalized to its total protein content determined by the Lowry method.

**Catalase (CAT)**

Cultures were washed with PBS, harvested in 300µL of RIPA buffer, and stored at -20 °C. Once thawed, the catalase assay kit was utilized per manufacturer’s instructions
(Cayman Chemical 707002). To sample wells, 30µL of methanol was added, followed by 20µL of hydrogen peroxide to initiate the reaction. After 20 minutes of incubation, 30µL of potassium hydroxide terminated the reaction. Next, purpald, a chromogen, was added to measure the colorimetric change due to formaldehyde formation. Subsequently, each reaction underwent a 5-minute incubation with 10µL catalase potassium periodate and the absorbance was read at 540nm. Catalase activity was determined from a 7-point catalase formaldehyde standard curve (provided by the kit). Catalase activity was standardized to its total protein content.

Caspase assay

Guava Caspase-3/7-FAM Kit (Millipore 4500-0540) was used to measure caspase-3/7 activity. Cell viability was simultaneously evaluated using 7-AAD. Cells were washed with 1X PBS and incubated with 10 µl of caspase reagent for 1 h at 37 °C in a 5% CO2 incubator. At the end of incubation, cells were washed with provided wash buffer, and incubated in 1X apoptosis buffer with 7-AAD for 10 min at room temperature. After incubation, cells were washed and changed to 1X PBS and analyzed with a Nikon fluorescent microscope.

qRT-PCR Apoptosis array

The RT² Profiler™ human Apoptosis qPCR Array (PAHS-012ZA, Qiagen) was used to measure the expression levels of 84 apoptosis-related genes (Table 5.1). Total RNA was isolated and quantified as previously described. A total of 0.5 µg of RNA was used for
cDNA synthesis and mixed with the kit supplied RT² SYBR Green Fluor qPCR Master Mix and distributed onto a 96-well array with a final concentration of 36.7 ng of cDNA per reaction. The qPCR was performed on a Bio-Rad iQ5 cycler according to the RT² Profiler PCR Array instructions under the following conditions: 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min. Five separate housekeeping genes (ACTB, B2M, GAPDH, HPRT1, RPLP0) within each array were used for normalization by calculating the ΔΔCₜ for each gene of interest. Fold changes of gene expression were analyzed by using RT² PCR array data analysis web portal (Qiagen, https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/) and a heatmap generated with heatmapper online software (Babicki et al., 2016).

**Induction of Apoptosis**

**Death Domain Receptors**: CRADD, FADD, TNF, TNFRSF10B (DR5).
**DNA Damage & Repair**: ABL1, CIDEA, CIDEB, TP53 (p53), TP73.
**Extracellular Apoptotic Signals**: CFLAR (Casper), DAPK1, TNFRSF25 (DR3).
**Other Pro-Apoptotic Genes**: BAD, BAK1, BAX, BCL10, BCL2L1, BID, BIK, BNIP3, BNIP3L, CASP1, CASP10 (MCH4), CASP14, CASP2, CASP3, CASP4, CASP6, CASP8 (FLICE), CD27 (TNFRSF7), CD70 (TNFSF7), CYCS, DFFA, DIABLO (SMAC), FAS (TNFRSF6), FASLG (TNFSF6), GADD45A, HRK, LTA (TNFB), NOD1 (CARD4), PYCARD (TMS1, ASC), TNFRSF10A (TRAIL-R), TNFRSF9, TNFSF10 (TRAIL), TNFSF8, TP53BP2, TRADD, TRAF3.

**Anti-Apoptotic**

**AKT1, BAG1, BAG3, BAX, BCL2, BCL2A1 (BFL1), BCL2L1 (BCLXL), BCL2L10, BCL2L2, BFA, BIRC3 (c-IAP2), BIRC5, BIRC6, BNIP2, BNIP3, BNIP3L, BRAF, CD27 (TNFRSF7), CD40LG (TNFSF5), CFLAR (Casper), DAPK1, FAS (TNFRSF6), HRK, IG1R, IL10, MCL1, NAIP (BIRC1), NFKB1, NOL3, RIPK2, TNF, XIAP (BIRC4).

**Regulation of Apoptosis**

**Negative Regulation of Apoptosis**: BAG1, BAG3, BCL10, BCL2, BCL2A1 (BFL1), BCL2L1 (BCLXL), BCL2L10, BCL2L2, BFA, BIRC2 (c-IAP1), BIRC3 (c-IAP2), BIRC6, BNIP2, BNIP3, BNIP3L, BRAF, CASP3, CD27 (TNFRSF7), CD40LG (TNFSF5), CFLAR (Casper), CIDEA, DAPK1, DFFA, FAS (TNFRSF6), IG1R, MCL1, NAIP (BIRC1), NOL3, TP53 (p53), TP73, XIAP (BIRC4).
**Positive Regulation of Apoptosis**: AKT1, BAG1, BAD, BAX, BCL2L1, BID, BIK, BNIP3, BNIP3L, CASP1 (ICE), CASP10 (MCH4), CASP14, CASP2, CASP4, CASP6, CASP8 (FLICE), CD40 (TNFRSF5), CD70 (TNFSF7), CIDE, CRADD, FADD, FASLG (TNFSF6), HRK, LTA (TNFB), LTB, NOD1 (CARD4), PYCARD (TMS1, ASC), RIPK2, TNF, TNFRSF10A (TRAIL-R), TNFRSF10B (DR5), TNFRSF25 (DR3), TNFRSF9, TNFSF10 (TRAIL-R), TNFSF8, TP53 (p53), TP53BP2, TRADD, TRAF2, TRAF3.

**Death Domain Receptors**

CRADD, DAPK1, FADD, TNFRSF10A (TRAIL-R), TNFRSF10B (DR5), TNFRSF11B (OPG), TNFRSF1A (TNFR1), TNFRSF1B, TNFRSF21, TNFRSF25 (DR3), TRADD.

**Caspases & Regulators**

**Caspases**: CASP1 (ICE), CASP10 (MCH4), CASP14, CASP2, CASP3, CASP4, CASP5, CASP6, CASP7, CASP8 (FLICE), CASP9, CFLAR (Casper), CRADD, PYCARD (TMS1, ASC).
**Caspase Activation**: AIF1 (PDCD8), APAF1, BAX, BCL2L10, CASP1 (ICE), CASP9, NOD1 (CARD4), PYCARD (TMS1, ASC), TNFRSF10A (TRAIL-R), TNFRSF10B (DR5), TP53 (p53).
**Caspase Inhibition**: CD27 (TNFRSF7), XIAP (BIRC4).

Table 5.1. Apoptosis-related genes examined with the RT² Profiler™ qPCR array.
**Calcium assay**

For quantification of extracellular matrix calcium, cells were harvested in radioimmunoprecipitation buffer (RIPA; 1XPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4, and 1:100 protease inhibitors) with extensive scraping using the pipet tip. Calcium deposition was determined based on calcium ions (Ca\(^{2+}\)) reacting with Arsenazo III (Genzyme, Canada) to form a purple Ca-Arsenazo III complex, which was measured at 655 nm. The concentration of total calcium in the sample was calculated based on a CaCl\(_2\) standard and calcium content was normalized to the total protein content of the sample using the Lowry method (Bio-Rad) as we have also done previously when screening with mouse cells (zur Nieden et al., 2004; zur Nieden et al., 2010a, b). This normalization is important to truly assess the differentiation level of the cultures without interference from cytotoxic or proliferative events. Calcium content was then standardized to solvent only controls and concentration-response curves charted. The half-maximal inhibitory dose (ID\(_{50}\)Ca) for each compound was taken from linear interpolation of the curve.

**Statistical analysis**

Data presented are averages of three independent experiments ± standard deviation. Significance was determined by using a web-based one-way ANOVA and Tukey HSD post hoc test (http://faculty.vassar.edu/lowry/anova1u.html) or unpaired Student's t-test as appropriate.
Results

Sidestream smoke reduces OCN expression in osteogenically differentiating hESCs

Prior evidence suggested that Camel and Camel Blue SS smoke and Camel STE inhibit in vitro differentiation of osteoblasts (Fig 5.1A). Here, we examined the expression levels of OCN, a gene that codes for the non-collagenous extracellular matrix (ECM) protein OCN, which is exclusively synthesized by osteoblasts and used as a marker for bone mineralization (Ducy and Karsenty, 1995) in osteoblasts differentiated from hESCs. The dose-response curve showed that Camel, Camel Blue, and STE gradually suppressed OCN expression with increasing concentrations (Fig 5.2A) supporting our earlier findings.

The inhibitory effect on osteogenesis manifested early at day 7 of the differentiation as osteoprogenitor genes were down-regulated at this early time point. For instance, the master regulatory transcription factors TWIST1 and RUNX2 were differentially expressed (Fig 5.2B). Specifically, effective doses of Camel Blue and STE resulted in the 1.79- and 1.62-fold upregulation of TWIST1 compared to the untreated control, respectively. RUNX2 was downregulated in Camel Blue and STE, to 0.58- and 0.68-fold respectively. We also

Table 5.2. Primer sequences used in this study. Ta, annealing temperature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Ta</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GAGTCAACGGATTTGTCGT-3'</td>
<td>5'-TTGATTTTGGAGGATCTCG-3'</td>
<td>60</td>
</tr>
<tr>
<td>SOD2</td>
<td>5'-GGCCAAAGGGAGATTTACAA-3'</td>
<td>5'-GGCTGTCAGCTTCTCCCTAAAAC-3'</td>
<td>60</td>
</tr>
<tr>
<td>Catalase</td>
<td>5'-CCCCAACTATTACCCCCAACAG-3'</td>
<td>5'-ATGTTCCTCACACAGGCCGTTC-3'</td>
<td>60</td>
</tr>
<tr>
<td>OCN</td>
<td>5'-GGCAGCGAGGTAGTGAAGAG-3'</td>
<td>5'-CTGGAGGAGGAGCAGAAGTGC-3'</td>
<td>60</td>
</tr>
<tr>
<td>TWIST1</td>
<td>5'-ACTGGCCCTGAAAAACCATAG-3'</td>
<td>5'-TGCAATTTACCATGGGTCC-3'</td>
<td>60</td>
</tr>
</tbody>
</table>
analyzed the expression of SATB2, a nuclear protein involved in osteoblast development. Developing mouse embryos show expression of SATB2 in the first branchial arch (Zhang et al., 2011; Dobreva et al., 2006) and SATB2 null mice have craniofacial defects (Dobreva et al., 2006). When assessed at E15.5, Satb2−/− tibiae showed reduced osteoblast mineralization. Camel Blue showed no regulation over SATB2; however, STE treated cultures had a 1.99-fold upregulation (Fig 5.2B).

**Figure 5.2.** Tobacco dysregulates the expression of osteogenic genes. (A) Concentration-response curves for differentiation (ID50) of OCN quantitative PCR. Dose-dependently, OCN mRNA expression decreased. (B-D) Osteogenic promoting genes showed HRTP-misregulation. (B) TWIST1 was significantly upregulated in ED cell. (C) RUNX2 mRNA was reduced and (D) SATB2 showed a 1.99-fold increase in STE effective dose. UNT, untreated; NED, non-effective dose; ED, effective dose; STE, Camel Snus tobacco extract; OCN, osteocalcin; TWIST1, Twist Family BHLH Transcription Factor 1; RUNX2, Runt-related transcription factor 2; SATB2, Special AT-rich sequence-binding protein 2. n = 3 independent samples ± SD. *p<0.05 one-way ANOVA versus untreated control.
Sidestream Camel smoke exposure causes apoptosis, while Sidestream Camel Blue and STE do not

Because the MTT assay performed as part of the tobacco hEST, suggested Camel to be cytotoxic (compare Fig. 5.1A), Caspases 3 and 7 activity levels were measured in an effective dose (ED) determined from the concentration response curves. Effective doses were defined as the doses at which the ECM calcium was reduced to 50%. A respective non-effective dose (NED, at 100-fold below the effective) was run alongside the effective dose in addition to an untreated control. On day 7 of differentiation, caspase 3/7 activation was observed only in the Camel ED showing more apoptotic cells compared to the untreated control, respective NED and all the HRTP-treated cultures (Fig 5.3).

We further aimed to understand the cytotoxicity of Camel SS compared to Camel Blue SS and STE treated cultures on the molecular level and hypothesized that alterations in gene expression of apoptotic mediators were causing apoptosis to occur in Camel ED. On day 7 of osteogenic differentiation, we employed a human apoptosis array, simultaneously analyzing 84 apoptosis-related genes. As shown

![Figure 5.3. Camel sidestream smoke induces apoptosis. Conventional Camel showed increased apoptosis determined by Caspase 3/7 activation. Camel Blue and Camel STE did not show apoptotic cells in effective doses (ED). Co-administration of ascorbic acid (AA) reversed apoptotic activity. hESCs, human embryonic stem cells; STE, Snus tobacco extract; NED, Non-effective dose; ED, effective dose; UT, untreated.](image-url)
in the heatmap (Fig 5.4A), our qPCR array results showed significant differential regulation of apoptosis-related genes in the Camel ED. The majority of the genes were upregulated. Further, hierarchical clustering showed that the effective doses of Camel Blue and STE did not have commonly affected genes, neither had they a close association to the genes regulated in Camel ED (Fig 5.4A). These data indicate potentially different pathways of apoptosis regulation.

Camel ED mRNA analysis resulted in significant upregulation of the following of pro-apoptotic genes (Fig 5.4B-D). In particular, the following 12 genes were significantly upregulated: ABL1, GADD45, CIDEA, CIDEB, DFFA, AIF, HRK, AIFM, CYCS, CASP9, BNIP3L, and BAX, which are pro-apoptotic genes associated with response to DNA damage and mitochondrial stress (Wang, 2000; Salvador et al., 2013; Fig 5.4B; Table 5.2). In contrast, Camel Blue ED treated cells showed upregulation of only a subset of these genes (AIF, CIDEB, AIFM, CYTC, and CASP9). The most striking difference was the specific regulation of ABL1 in Camel ED. Camel STE upregulated 3 genes out of the 12: CIDEA, AIF, and AIFM. In addition, both Camel Blue and STE upregulated CASP4 mRNA, while Camel SS did not (Fig 5.4E). While the activation of most caspases is associated with apoptosis, caspase 4 has been shown to respond specifically to endoplasmic reticulum (ER) stress (Hitomi et al., 2004), potentially indicating that osteogenic inhibition in HRTP-treated cultures could be partially due to ER stress.

To validate the finding that Camel SS exposure induced apoptosis by inducing mitochondrial stress, we measured the mitochondrial membrane potential, which is determined by the flux through the electron transport chain and generation of a proton
gradient across the membrane. This was done using JC-1, a cationic dye that enters and accumulates in the mitochondrial matrix and forms j-aggregates. J-aggregates fluoresce red and indicate an increase in membrane potential. Strong JC-1 aggregates, an indication of a healthy cell, were observed in all non-effective doses, HRTP effective doses, and control cultures (Fig 5.4C). In contrast, Camel effective dose-treated cells had diminished aggregates and increased monomers, as JC-1 remained in its monomeric form as determined by its green fluorescence. Suggesting that the mitochondrial membrane potential collapsed, and the cells were in a state of stress. This data indicated that exposure to Camel SS smoke can result in mitochondrial dysfunction.

*Inhibition of osteogenesis is linked to oxidative stress*

Next, we investigated whether oxidative stress, a common link between toxicant exposure and cytotoxicity, was responsible for the observed adverse outcomes associated with tobacco exposure. To causally address this, caspase 3/7 activation was measured in cells treated with ascorbic acid (AA) concomitantly with exposure to an effective dose. As expected, if apoptosis were to occur via oxidative stress, the presence of AA diminished the caspase 3/7 signal in the Camel SS ED (Fig 5.3).

Co-administration of tobacco products and antioxidants (i.e., AA, vitamin E (VE), and glutathione reduced ethyl ester (GSH-OEt) reestablished calcium deposition to control levels (Fig 5.5A). These results suggest that excessive ROS may lead to cytotoxicity via apoptosis due to the activation of caspase 3/7.
Figure 5.4. Camel tobacco products de-regulate apoptosis-related genes. A) Clustering of 84 apoptotic genes transcriptionally responsive between non-effective (NED) and effective (ED) doses of Camel, Camel Blue, and Camel Snus tobacco extract (STE) as measured with the RT² Profiler™ qPCR array. Camel clustered away from the other treatments with positive regulation of apoptosis. Each row represents the relative expression of a single gene. B) Graphs show the overview of significantly upregulated apoptotic genes categorized by apoptotic response. (C) Reduced mitochondrial membrane potential measured by JC-1 dye is a consequence of Camel toxicity. Mitochondrial permeability was not affected by HRTPs. n = 3 independent samples ± SD. *p < 0.05 one-way ANOVA versus untreated control (UNT).
HRTPs generate subtoxic levels of ROS

Oxidative stress is the imbalance between reactive oxygen species (ROS) and antioxidants. Therefore, we next investigated which specific ROS species were responsible for observed oxidative stress. ROS are highly reactive molecules that consists of radical and non-radical oxygen species such as superoxide anion ($O_2^-$), hydroxyl radical ($OH^-$), and hydrogen peroxide ($H_2O_2$). $O_2^-$ is the primary ROS produced and quickly turned over to generate the more aggressive secondary ROS ($H_2O_2$ and $OH^-$; Sharma et al., 2012; Circu and Aw, 2010). Mainstream smoke solutions did not generate significant levels of $O_2^-$. However, we confirmed that effective doses of Camel and Camel Blue SS smoke and STE generated $O_2^-$ in H9 cells differentiating into osteoblasts on day 7 (Fig 5.6A), which is

![Image of bar graphs](image)

**Figure 5.5.** Tobacco exposure decreases antioxidant activity. Effective (ED) doses of Camel, Camel Blue, and Camel Snus tobacco extract (STE), determined by dose response curves (compare prior data), were compared against non-treated controls. Co-administration of antioxidants ascorbic acid (AA), vitamin E (VE), or glutathione reduced ethyl ester (GSH-OEt) with its respective ED of tobacco product (A) rescued osteoblast inhibition and (B) diminished $O_2^-$ production. hESCs, human embryonic stem cells. $n = 5$ technical replicates for calcium assay, $n = 3$ independent samples for $O_2^-$ determination $\pm$ SD. *p<0.05 one-way ANOVA versus untreated control. UNT, untreated; $O_2^-$, superoxide anion.
when the osteoprogenitor genes were deregulated (compare Fig. 5.2B). The highest increase in superoxide anion content was registered in the Camel SS exposed cells, followed by the Camel Blue SS exposed cells and then STE. Effective doses of differentiating cells treated with antioxidants alleviated O$_2^-$ levels (Fig 5.5B.) In addition, DHR123 measured a significant increase in H$_2$O$_2$ on day 7 in all treatments (Fig 5.6B).

ROS are a natural byproduct generated through the activation of enzymes such as NADPH oxidase (NOX), a membrane enzyme, and mitochondrial oxidases (Circu and Aw, 2010). Hence, NOX activity was next assessed by the lucigenin chemiluminescence method. NOX activity was increased in effective doses over relevant non-effective doses on day 7 of differentiation with a 1.68, 1.8, and 2.04-fold increase by 3 min and 1.73, 2.32, and 1.71-fold increase at 30 min in the order of Camel ED, Camel Blue ED and Camel STE, respectively (Fig 5.6C).
Another major source of $O_2^\cdot$ generation is in the mitochondria. The tobacco-induced increase in mitochondrial superoxide anion was confirmed by MitoSOX for Camel SS. However, mitochondrial ROS production was not affected by the HRTPs Camel Blue and STE (Fig 5.6D), indicating mitochondrial dysfunction was occurring in the Camel treated cultures only. These data suggest that the observed cytotoxicity of Camel SS may be due to excessive levels of $O_2^\cdot$ formed by the mitochondria with a secondary role for NOX. In contrast, NOX appear to be the major contributor of $O_2^\cdot$ in the HRTP-treated cells. Therefore, the detrimental effect of the HRTPs on osteogenic differentiation can potentially be attributed to subtoxic levels of $O_2^\cdot$ that appear to support cell survival, but instead inhibit differentiation along the proper lineage.

_Tobacco-induced decrease in antioxidant enzyme activity_

When the equilibrium between ROS and antioxidant levels is disrupted, oxidative stress occurs. To assess the presence and/or lack of endogenous antioxidant contributions by antioxidant enzymes, enzyme activity levels of superoxide dismutase (SOD), the first ROS detoxifying enzyme, and catalase (CAT) were measured. SOD is a free scavenging enzyme that dismutates $O_2^\cdot$ into $H_2O_2$. In turn, $H_2O_2$ is degraded to water and molecular oxygen by the enzyme CAT. Interestingly, there was a significant reduction in SOD and catalase activity (Fig 5.7A), which was evident at the mRNA level for SOD2, but not CAT (Fig 5.7B).
Discussion

Earlier work from our lab has shown that administration of tobacco products decreases osteoblast production during differentiation. However, the mechanisms responsible for osteogenic inhibition have not been fully elucidated. This present study shows that tobacco products increase ROS in H9 hESCs as they are differentiating into osteoblasts. Our findings further suggest that NOX and the mitochondrial electron transport chain are contributing sources of ROS production. Other enzymes, such as xanthine oxidase can also produce superoxide anion, but only a small amount of ROS are generated by the normal activity of xanthine oxidase. However, conditions like ischemia have been attributed to high ROS levels generated by xanthine oxidase (Ahmad et al., 2017). During oxidative stress, membrane lipids are also susceptible to oxidation by ROS. The oxidation results in the formation of a lipid radical and initiating lipid peroxidation (Phaniendra et al., 2015). Lipid peroxidation disrupts membrane integrity and plays a role in various
pathological conditions such as cardiovascular diseases and neurodegeneration (Nam, 2011). These other generators of ROS such as lipid peroxidation and xanthine oxidase need to be further explored to determine the main contributor(s) of ROS in the HRTP-mediated inhibition of skeletal development. Tobacco exposure attenuated the activity of SOD and CAT. Importantly, osteogenic inhibition was reversed by supplementation of antioxidants during differentiation causally linking tobacco exposure, oxidative stress and inhibition of osteoblast development.

Oxidative stress can occur due to aging and exposure to radiation, drug therapies, and environmental toxicants (Valko et al., 2007; Tilg et al., 2008). Oxidative stress can lead to the damage of cell membranes, DNA, proteins, and enzymes (Birben et al., 2012), which can result in damage to organs. Bone diseases such as osteoporosis have been attributed to oxidative damage. For example, osteoporosis in postmenopausal women has been shown to associate with activation of NOX coupled with reduced function of antioxidant enzymes (Sendur et al., 2009). In skeletal disorders that cause low bone mineral density, studies indicate oxidative stress to have a role in the pathogenesis of the disease (Tilg et al., 2008). It has also been suggested that oxidative stress supports osteoclast differentiation and activity through the upregulation of RANKL and induces osteoblast apoptosis (Tilg et al., 2008; Guo et al., 2017). Both osteoblast reduction and increased osteoclast activity would disrupt osteogenesis and perpetuate decreased bone mass making bones fragile and susceptible to fractures.

The combustion of a cigarette can produce excessive levels of ROS that would not be present in the tobacco leaf or ash alone (Huang et al., 2005). Cigarette filters are not
equipped to remove the ROS. The gas and solid phase of SS smoke has high levels of free radicals (Valavanidis et al., 2009). Further, water-soluble components can produce $O_2^-$ and subsequently $H_2O_2$, showing that the involuntary exposure to cigarette smoke (passive smoking) is a health hazard to vulnerable populations such as the developing fetus, infants, and young children. Smokeless tobacco has also been demonstrated to induce oxidative stress and lead to extensive DNA damage and potentially cancers of the mouth (Bagchi et al., 2001; Katakwar et al., 2016). It is therefore plausible, that 1) chemicals other than combustion products only may be causes of health issues, and that 2) tobacco-induced oxidative stress can target the bone specifically to cause disorders. Yet, it remains to be uncovered if oxidative stress can contribute to tobacco-associated skeletal birth defects.

The degree of elevation of ROS can have different effects on the vitality of the cell. At low levels, ROS generation could be beneficial and necessary for homeostasis. ROS produced by macrophages have been shown to suppress T cell-dependent arthritis (Gelderman et al., 2007). Importantly, low levels of ROS can support hematopoietic stem cell (HSC) self-renewal and proliferation. With increased levels of ROS, cell cycle inhibitors such as p16INK4 are activated to stop HSC self-renewal without damage to the cell (Ito et al., 2006). In this study, the data suggest that excessive levels of ROS result in cytotoxicity as seen in Camel treated cultures. Conversely, Camel Blue and STE generate ROS at lower levels that do not lead to cytotoxicity but results in a HRTP-mediated skeletal differentiation defect. This notion was further supported by the high apoptotic activity in Camel treated cultures as uncovered with the apoptosis array, which showed upregulation of pro-apoptotic and DNA damage genes. In the HRTP cultures instead, only a small subset
of such genes was upregulated. In particular, the upregulation of CASP4 in the HRTP exposed cells may point towards differentiation inhibition through an inflammation/ER-stress response. Therefore, conventional and harm-reducing tobacco products might show different modes of bone inhibition.

The hypothesis that oxidative stress is a major modulator of development is relatively new but has already been examined for a variety of chemicals. With recently increased interest in redox biology and disease in the past few years, teratogenic agents such as thalidomide, benzo[a]pyrene, and phenytoin have been linked to ROS-mediated teratogenesis. Specifically, the action of thalidomide, an agent known to cause limb defects in humans and rabbits, can be reversed with pre-treatment of the free-radical spin trapping agent phenylbutylnitroné (Lee et al., 2011). The attenuation of osteogenesis in this case can thus potentially be attributed to ROS at levels that did not elicit cytotoxicity and is reversible with the up-regulation of free-radical scavenging enzymes, as also shown here.

Other studies have reported a ROS-mediated reduction in adult osteogenesis (Almeida et al., 2007; Ambrogini et al., 2010; Iyer et al., 2013). In contrast to our work, these previous studies look at the role of ROS on adult stem cells, such as mesenchymal stem cells (MSCs). Results obtained with adult stem cells can be indicative of age-related disorders, such as osteoporosis, however fail to address how the tissue originally develops. In contrast, our study demonstrates skeletal defects during embryonic development in association with oxidative stress. Specifically, it is the use of human ESCs that allows for the assessment of differentiation defects that are directly relevant to the developing human embryo and fetus.
In summary, we have demonstrated that the HRTPs Camel Blue and STE are detrimental to the development of the embryonic skeleton. We suggest the underlying mechanism of HRTP osteoblast inhibition, in part, be due to the generation of sub-toxic ROS levels. How this sub-toxic oxidative stress may potentially cause differentiation inhibition molecularly will be addressed in the following chapter.
References


10. Byron MJ, Jeong M, Abrams DB, Brewer NT. Public misperception that very low nicotine cigarettes are less carcinogenic Tobacco Control Published Online First: 23 January 2018.


Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2006.


56. zur Nieden NI, Davis LA, Rancourt DE. Monolayer cultivation of osteoprogenitors shortens duration of the embryonic stem cell test while reliably predicting developmental osteotoxicity. Toxicology. 2010b;277(1-3):66-73.


Harm-reduction tobacco alters bone development through tobacco sensitive transcriptional regulators

Nicole Renee Lee Sparks and Nicole Isolde zur Nieden

Introduction

Transcriptional regulation of skeletal development is an important step for the correct expression of genes to determine the fate of a developing cell. Even though osteoblasts arise from mesenchymal cells of neural crest or mesoderm origin, each of the pathways to generate that mesenchymal cell has their own regulatory expression profile. Misregulation of these transcription factors has been identified to play a role in hypoplastic clavicles, various dental defects, delayed ossification of the skull bones, premature fusion of the skull sutures, and a number of other skeletal defects (Jensen et al., 2010). Because of their importance, alteration of such regulatory genes due to toxicant exposure can be detrimental to skeletal development. As previously discussed (chapter 4), toxicants can inhibit osteogenic differentiation of human embryonic stem cells (hESCs) and neural crest (NC)- and mesoderm-derived osteoblasts can respond differently to chemical agents during differentiation. Our prior work has also shown that when osteogenically differentiating hESCs are exposed to popular brands of tobacco products, their differentiation potential is decreased. Maternal smoking has been shown to be detrimental to bone development of
newborns (Carmichael et al, 2008; Kawakita et al., 2008; Karatza et al., 2003). However, it remains to be uncovered if regulatory genes of osteoblast differentiation are tobacco-sensitive.

While many signaling cascades influence transcription factors, oxidative stress can also disrupt transcriptional activity of genes essential for regulating the transcriptional profile of osteogenesis. As outlined in the last chapter, cells are equipped to counteract ROS and prevent oxidative stress through various mechanisms including the actions of free-scavenging enzymes SOD and CAT. This response requires the activation of the redox-sensitive forkhead foxO (FOXO) family of transcription factors. Therefore, we hypothesized that FOXOs may be misregulated upon tobacco exposure ultimately diminishing the ability to remove ROS.

The FOXO family is evolutionarily conserved with a vital role in cell cycle control, longevity, and regulation of redox mechanisms. Members of the FOXO family include FOXO1, FOXO3a, FOXO4, and FOXO6 in humans (Huang and Tindall, 2007). FOXO1 and 3 have similar expression patterns in adult tissue, while FOXO6 is specific to the brain (van der Vos and Coffer, 2008). *Drosophila melanogaster* and *Caenorhabditis elegans* studies first identified FOXO and named it dFOXO and DAF-16, respectively (Huang and Tindall, 2007). *Drosophila melanogaster* and *Caenorhabditis elegans* have shown that the transcription factors are controlled by the evolutionary conserved insulin-phosphotidylinositol 3 kinase-protein kinase B (insulin-PI3K-PKB) signal transduction cascade, which promotes the nuclear exclusion of FOXO (Essaghir et al., 2009). To be active, FOXO is post-translationally modified through phosphorylation/
dephosphorylation, methylation, ubiquitination, and acetylation/deacetylation (Brown and Webb, 2017). The same mechanisms can regulate FOXO negatively with the addition of miRNAs (Brown and Webb, 2017).

Reports have linked FOXO regulation in oxidative stress-induced osteoblast inhibition. In the absence of FOXO3, bone mass in cortical and cancellous bone was decreased with increased oxidative stress in 3-month-old mice. In a mouse triple knockout of FOXO 1/3/4, enhanced apoptosis and bone loss was observed (Ambrogini et al., 2010). Further, overexpression of FOXO3 in adult osteoblasts supported bone formation, osteoblast yield, and reduced oxidative stress. While these cases demonstrate the role of FOXO in adult bone homeostasis, it is unknown if such mechanisms are at play during embryonic skeletal development. In this study, we investigate the role of FOXO during osteoblast differentiation using human embryonic stem cells (hESCs) investigating a link between tobacco exposure, FOXO regulation and downstream gene activation.

Materials and Methods

Human ESC maintenance and differentiation.

Human ESCs were maintained in mTeSR medium as previously described. Briefly, osteogenesis was initiated on confluent colonies with DMEM and 15% FBS for the first 5 days of differentiation. At that time, β-glycerophosphate, ascorbic acid, and 1,25(OH)2 Vitamin D3 were added to the culture medium for the remainder of the differentiation (previously described). Throughout differentiation, cells were exposed to either non-effective doses (NED) or effective doses (ED) of Camel SS, Camel Blue SS, or Camel
STE. The FOXO concentration-response curve was established from 7 concentrations with an untreated control. Smoke solutions were replenished with each media change.

Neural crest cell induction

H9 hESCs colonies maintained in mTeSR were dissociated with accutase and plated as singles cells in differentiation medium (DMEM/F12, 2% B27 supplement [ThermoFisher], 1x Glutamax [Gibco], 0.5% bovine serum albumin). ROCK inhibitor Y27632 (10 µM Tocris Bioscience) was added for the first 24 hrs. CHIR 99021 (3 µM Tocris Bioscience) was added to the medium for the first two days.

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde for 30 min at 4°C and permeabilized with 0.1% Triton-X 100 for 15 min, cells were incubated in primary antibody diluted in PBS/10% FBS overnight at 4°C. Primary antibodies used were FOXO1 (ab39670), FOXO3a (ab23683), SOX10 (Santa Cruz, SC17342), and TFAP2α (C83E10 Cell Signaling). Cells were then washed and incubated in the appropriate secondary antibody (AlexaFluor conjugated) and DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) for 2h. Cells were observed on a Nikon Eclipse Ti-S fluorescent microscope.

**Histochemical analysis**

Cultures were washed with PBS and fixed in 4% paraformaldehyde at 4°C for 30 min. For Von Kossa stain, fixed samples were overlaid with silver nitrate solution (Ricca
Chemical Company) and illuminated under an intense light source for 1 h. Cultures were then incubated with 5% sodium thiosulfate (Red Bird Service) for 2 minutes to visualize the von Kossa stain. Cells were also washed with PBS and overlaid with 2% w/v Alizarin Red S solution (Sigma-Aldrich) for 5 minutes to visualize calcium-rich areas. Prior to imaging, cultures were washed 3X with water followed by ascending alcohol washes (70%, 80%, 90%, 100% ethanol).

**RNA isolation and quantitative PCR**

Total RNA was extracted with the NucleoSpin RNA II protocol (Macherey Nagel). RNA concentration was determined with a NanoDrop® 1000 spectrophotometer (Thermo Scientific) at 260 nm. 25 ng of total RNA was used for cDNA synthesis with a mastermix including 1X reaction buffer, 0.5 mM dNTPs, 20 U/µL RNase inhibitor, 0.8 U/µL reverse transcriptase, and 1 µM random primer. 25ng cDNA transcripts were used for quantitative polymerase chain reaction (qPCR) with SYBR green on the MyiQ cycler (BIO-RAD). Reactions were setup for 10 minutes of denaturing at 94°C, followed by 40 cycles of denaturing at 94°C, and annealing/elongation at 60°C for 45 seconds. The n-fold expression in target samples was calculated with the ΔΔCT method by standardizing CT values to GAPDH expression (Table 5.1).

**Protein extraction**

On day 7 of differentiation, whole cell, nuclear, and cytoplasmic protein were collected. Prior to collection, cell cultures were pretreated with 1mM sodium orthovanadate
(stable for 30 minutes) for 30 minutes to inhibit protein tyrosine phosphatases (PTPs), which regulates the phosphorylation state of multiple signaling molecules. This pre-treatment preserves the phosphorylation of proteins. Whole cell collections were carried out with RIPA buffer (RIPA: 1XPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4, 1:100 phosphatase inhibitors, and 1:100 protease inhibitors)) with Halt protease and phosphatase inhibitor cocktails (Thermo Scientific) and stored at -80°C. Nuclear and cytoplasmic fractions were extracted with the NE-PER Nuclear and Cytoplasmic Extraction kit as per the manufacturer’s instructions. Harvested cultures were lysed in CER I buffer provided with the kit (including protease inhibitors, sodium orthovanadate, sodium fluoride, and phenylmethylsulfonyl fluoride. Samples were centrifuged for 5 minutes at maximum setting (~16,000g) and the supernatant extracted and stored at -80°C (cytoplasmic portion). The pellet was resuspended in NER buffer provided with the kit (including protease inhibitors, sodium orthovanadate, sodium fluoride, and PMSF). For 40 minutes samples were intermittently vortexed, then centrifuged for 10 minutes to collect the supernatant (nuclear content) and stored at -80°C. Protein concentrations were determined by the Lowry method described in chapter 2.

Western Blot analysis

Equal amounts of protein were loaded into a 10% SDS/polyacrylamide gel and ran at 100V for 1 hour. Proteins were transferred from gels onto PVDF membranes at 30V in a transfer chamber overnight at 4°C, apparatus contained 1X transfer buffer (100 mL 10X transfer buffer [30.2 g Tris-Base, 188 g Glycine, and 1 L ddH₂O], 200mL methanol, and
700ml ddH$_2$O). Membranes were blocked in 5% BSA/TBST-T for 60 min. The membranes were then at 4°C overnight in one of the following primary antibodies: rabbit anti-FOXO1 (ab39670 Abcam), rabbit anti-FOXO3a (ab23683 Abcam), mouse anti-β-catenin (Thermo Scientific), mouse anti-TATA binding protein (Abcam), and mouse anti-Tubulin (Abcam). Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology) was used as a secondary antibody diluted in 10% FBS/PBS. Enhanced chemiluminescence (SuperSignal West Pico Chemiluminescence) was used to detect immunoreactive proteins on the Bio-Rad Gel Doc system.

**Immunoprecipitation analysis**

Three hundred micrograms of nuclear lysates were subjected to immunoprecipitation with 2 µg of β-catenin primary antibody. Proteins bound to protein A-sepharose CL-4B beads (GE Healthcare) were washed and equal amounts of protein, determined by beta-catenin Western blot, were loaded into a 10% SDS/polyacrylamide gel. Gels were blotted onto PVDF membranes as described in Western blot protocol (above), incubated with FOXO1 or FOXO3 primary antibodies and subsequently with secondary antibodies for chemiluminescence visualization.

**shRNA expression vector construct**

FOXO shRNA 21 bp sequences were determined from MISSION® shRNA Plasmid DNA (Sigma Aldrich). For vector construction, BamHI (5’) and HindIII (3’)
restriction sites were included at the end of the oligo. A loop sequence with an XhoI restriction site was inserted between the sense and antisense sequences. Table 6.1 contains the oligonucleotide sequences, where green indicates the sequence for insertion into the BamHI or HindIII restriction enzyme sites, and red indicates the short hairpin loop sequence. The sense shRNA sequence is in bold lettering and the antisense strand follows after the loop sequence. Two shRNAs were designed for each FOXO of interest. Forward (45 µM) and reverse (45 µM) oligos were annealed in 1X annealing buffer (10x annealing buffer: 1M K-acetate, 0.3 M HEPES-KOH pH7.4, and 20 mM Mg-acetate). Annealing was carried out in a thermocycler set to 95°C and programmed to reduce by 1°C every minute until the reaction reached 25°C. 1 µg of the cloning vector, pBK-Flex-shRNA (Fig 6.1), was double digested with 1.0 unit of BamHI and 1.0 unit of HindIII in 50 µL reaction volume (1X NEB Buffer 2.1 New England BioLabs) at 37 °C for 1.5 hours. The digested cloning vector was resolved on a 1% agarose gel and purified using the GeneJet PCR Purification kit (ThermoFisher). The annealed oligos were ligated overnight at room temperature to the digested cloning vector in a 10

Figure 6.1. pBK-Flex-shRNA plasmid map. shRNA of FoxO1 or FoxO3 was inversely inserted at BamHI and HindIII restriction sites. shRNA, short hairpin RNA.
μL reaction volume (2 μL cloning vector, 2 μL annealed oligonucleotides, 1X ligation buffer, and 1.0 unit of ligase).

*Bacterial transformation*

The ligation mixture (5 μL) was transformed into Stbl2™ competent cells (ThermoFisher). 50 μL aliquots of Stbl2 stored at -80 °C were thawed on ice. The ligated DNA was added to the competent bacterial cells and incubated on ice for 5 minutes. The DNA-bacterial cell mixture was heat showed for 30 seconds at 42 °C and then placed back on ice for 2 minutes. 500 μL of Luria-Bertani (LB) broth was added to the mixture and then incubated for 30 minutes at 37 °C. The mixture (100 μL) was spread onto ampicillin (100 μg/mL) LB agar plates and incubated overnight (12-18 hours) at the 37 °C. Individual colonies were selected and grown in 5 mL of LB Broth with ampicillin overnight in a shaking incubator at 37 °C set to 175 RPM. After incubation, the plasmid DNA was purified using the E.Z.N.A.® Plasmid Mini Extraction Kit (Omega Bio-tek). Purified plasmids were restriction enzyme digested with 1.0 unit of XhoI in 1X CutSmart® Buffer (New England BioLabs) and resolved on a 1% agarose gel. Plasmids that yield two products at 6.6 kb and 1.6 kb had the correct insertion of the FOXO shRNA into the vector.

*Generation of cre-inducible FOXO shRNA stable cell lines*

1 μg of pBK-Flex-FOXO1-1 (sequence 1), pBK-Flex-FOXO1-2 (sequence 2), pBK-Flex-FOXO3-1 (sequence 1), and pBK-Flex-FOXO3-2 (sequence 2) plasmids were linearized with 1.0 unit NotI restriction enzyme in 1X NEBuffer 3.1 buffer. The digested
plasmids were purified using the GeneJet PCR Purification kit. Single cell human ESCs in mTeSR were transfected with 200 nanograms of linearized pBK-Flex-FOXO1-1, pBK-Flex-FOXO1-2, pBK-Flex-FOXO3-1, or pBK-Flex-FOXO3-2 using the Effectene Transfection Reagent (Qiagen). 72-96 h after transfection, individual colonies were selected and plated into a 96-well format. The individual colonies are now referred to as clones with a denoted number. Once confluent, the clones were expanded to a 48-well format and half of the culture was assessed for the presence of GFP by PCR analysis. On day 5 of differentiation, FOXO shRNA clones were transfected with 500 ng of Cre recombinase, generously donated by Dr. Martin Riccomagno, University of California Riverside. In this Cre-dependent system, the absence of Cre keeps the shRNA in an antisense orientation—the system off. In the presence of Cre, the cassette flips and results in a sense “on” orientation.

<table>
<thead>
<tr>
<th>FOXO1-1</th>
<th>GATCCatctacagtggatgtgtaaACTCGAGAttgacatcactgtagatTTTTTGGAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>FOXO1-1 Reverse</td>
</tr>
<tr>
<td></td>
<td>AGCTTCCAAAAAAAtactacagtggatgtgtaaACTCGAGAttgacatcactgtagatG</td>
</tr>
<tr>
<td>FOXO1-2</td>
<td>GATCCgcggagtttagcagtctcaaaACTCGAGAttgagctgtaaactcggGTTTTTGGAA</td>
</tr>
<tr>
<td>Forward</td>
<td>FOXO1-2 Reverse</td>
</tr>
<tr>
<td></td>
<td>AGCTTCCAAAAAAGccggagtttagcagtctcaaaACTCGAGATTtgagctgtaaactcggG</td>
</tr>
<tr>
<td>FOXO3-1</td>
<td>GATCCggacaataacgaaacttacACTCGAGAgtactactgcttctgtaaacGTGGTGGGA</td>
</tr>
<tr>
<td>Forward</td>
<td>FOXO3-1 Reverse</td>
</tr>
<tr>
<td></td>
<td>AGCTTCCAAAAAAGggacaataacgaaacttacACTCGAGATTtgactctgcttctgtaaacG</td>
</tr>
<tr>
<td>FOXO3-2</td>
<td>GATCCcatgttcaatgagcttgtgaACTCGAGAtccagctccattgaacatgtTTTTTGGGA</td>
</tr>
<tr>
<td>Forward</td>
<td>FOXO3-2 Reverse</td>
</tr>
<tr>
<td></td>
<td>AGCTTCCAAAAAACatgttcaatgagcttgtgaACTCGAGATTccagctccattgaacatgtG</td>
</tr>
</tbody>
</table>

**Table 6.1.** Oligonucleotide sequences.
**Dot Blot analysis**

FOXO shRNA clones grown in a 48-well format were lysed in RIPA buffer (RIPA; 1XPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4, and 1:100 protease inhibitors) and 2 µL of protein lysates were spotted on strips of PVDF membrane grids. The membranes were dried and blocked in 5% BSA in TBS-T buffer for 30 min. Blots were incubated in FOXO1, FOXO3, or Tubulin primary antibodies for 30 min. HRP-linked secondary antibodies were incubated for 30 min. SuperSignal West Pico Chemiluminescence was used to detect immunoreactive proteins on the Bio-Rad Gel Doc system.

**RNA isolation, library preparation, and sequencing**

Total RNA of HRTP treated or control cultures were extracted with the NucleoSpin RNA II protocol. 100 ng of each RNA sample was used as input for RNA sample preparations. All samples passed initial quality testing using Bioanalyzer 2100 (Agilent), assuring RIN values > 9. Ribosomal RNA was depleted using the NEBNext® Poly(A) mRNA Magnetic Isolation Module. The libraries were constructed using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina. Libraries were submitted for 75-bp single-end sequencing by Illumina HiSEQ 2000 at the Genomics Core in the UCR Institute of Integrated Genome Biology (IIGB). A total of three samples for each time course (each sample in triplicate) were multiplexed and sequenced in one lane, each of which yielded ~240 million reads (~27 million reads per sample).
Single-end sequencing reads were uploaded to the Galaxy web platform (usegalaxy.org) (Afgan., et al., 2016) for analysis. The human reference genome (GRCh38/hg38) was aligned to the reads with the Tophat v2.1.0 algorithm (Trapnell et al., 2009). The data aligned by Tophat were processed by Cufflinks (Trapnell et al., 2010) to assemble transcripts and to measure their relative abundance in fragments per kilobase of exon per million fragments mapped (FPKM). Assembled transcripts from experimental samples were compared with the annotated transcriptome (GRCh38.89) downloaded from UCSC genome browser and examined for differential expression using Cuffdiff. The following criteria were used to select differentially expressed genes: (i) a fold change (FC) of at least 1 or more and (ii) a false discovery rate (FDR) threshold of 5%. Principle component analysis was generated in R on rlog (Love et al., 2014) transformation on the normalized count data of the differentially expressed genes. Heatmaps were generated also in R with the rlog transformation and clustered based on Pearson correlation. Gene ontology (GO) analysis was performed using DAVID Bioinformatics Resources 6.8 (Huang et al., 2009a, 2009b). GO terms were submitted to REVIGO to make a graph-based summary of the differentially regulated genes (Supek et al., 2011). Cytoscape software v3.6.1 was used to construct GO term networks from the REVIGO summary (Shannon et al., 2003). VENNY 2.1 (Oliveros, 2007-2015) was used to compare lists of genes in the different treatment cultures.
**Statistical analysis**

Data presented are averages of three independent experiments ± standard deviation. Significance was determined by using a web-based one-way ANOVA and Tukey HSD post hoc test (http://faculty.vassar.edu/lowry/anova1u.html) or unpaired Student's t-test as appropriate.

**Results**

**FOXO1 and FOXO3 activity increases during osteogenesis**

Published studies have shown that FOXO is activated and necessary for osteoblast maintenance (Iyer et al., 2013). However, it is not clear if FOXOs are needed during the

![Figure 6.2](image-url)

**Figure 6.2.** FoxOs are expressed during osteogenic differentiation. A) Time-course of FoxO1 and FoxO3 expression during osteogenic differentiation assessed by immunocytochemistry. FoxO presence correlates to neural crest cell-mediated osteogenic differentiation. FoxO1, FoxO3, and SOX10 positive cells appeared on day 7 of differentiation. B) FoxO (green), SOX10 (orange), and TFAP2α (red) with DAPI (blue) immunostaining on day 5 neural crest cells differentiated from hESCs (Leung et al., 2016). hESCs, human embryonic stem cells; TFAP2a, transcription factor AP-2 alpha; Sox10, SRY-box 10. Scale bar = 100 µm
entire differentiation process beginning with a pluripotent cell. To determine the presence of FOXOs during early osteogenic differentiation, we performed immunocytochemistry on hESC osteogenic cultures using time points corresponding to germ layer specification and lineage determination (Fig 6.2). Both FOXO1 and FOXO3a appeared to be transiently expressed during these early stages of cellular commitment. They were present in the nucleus as cells exited from pluripotency (day 1), but then down-regulated after.

Previous work has demonstrated that our differentiation protocol predominantly gives rise to osteoblasts of the neural crest lineage and to a lesser degree to mesoderm (chapter 2). After the addition of osteogenic factors to the medium, FOXOs were concomitantly expressed with SOX10 on day 7, indicating that both FOXO isoforms are found in neural crest cells. When using a 5-day neural crest induction protocol (Leung et al., 2016, described in chapter 2), we found cells that were triple-positive for FOXO1 or FOXO3 and the neural crest markers SOX10 and TFAP2α. These data suggest that FOXOs may play a role during osteogenic differentiation and, at least in part, during neural crest-associated osteogenic differentiation.

**HRTP represses the expression of FOXO1 and FOXO3**

As it became evident that FOXOs are expressed during in vitro osteogenesis, we next asked whether FOXO transcription was altered by HRTPs (Fig 6.3A). The osteogenically differentiating cultures were treated with different concentration of HRTPs and harvested on day 7, the time point that was previously found to show altered antioxidant
enzyme activity as well as ROS accumulation (see chapter 5). A dose-response curve shows a concentration-dependent decrease in FOXO mRNA levels, similar to that found for RUNX2 and TWIST1 (Fig 5.1A). Indeed, the effective doses of HRTPs chosen throughout this study decreased FOXO mRNA levels (Fig 6.3B). However, the cytotoxic conventional Camel SS did not have a regulatory effect on the transcription of FOXO.

HRTP excludes FOXO from the nucleus.

We next determined whether the HRTP-mediated reduction in FOXO mRNAs manifested at the protein level. While cytoplasmic and whole protein levels did not show a misregulation (Fig 6.4 A, B), FOXO1 and FOX3 protein levels were markedly reduced in the effective doses of Camel Blue and Camel STE (Fig 6.4B), suggesting that FOXOs were
inhibited from transcriptional activation upon HRTP exposure. Overall, this finding may suggest why the *SOD2* and *CAT* mRNAs were down-regulated in HRTP-treated cells.

A co-factor for FOXO1 and 3 to activate the oxidative stress defense targets *SOD2* and *CAT* is β-catenin (CTNNB1) (Iyer et al., 2013; Almeida et al., 2007). In addition, the Wnt/CTNNB1 signaling pathway plays an essential role in differentiation during skeletal development (Ding et al., 2012; zur Nieden et al., 2007). Specifically, in the absence of CTNNB1, craniofacial development is inhibited, and long bones are under-mineralized and shortened (Brault et al., 2001; Nishimoto et al., 2015).

To determine whether FOXO absence affected CTNNB1 binding in differentiating osteoblast, we examined if the two proteins interact in the nucleus. FOXO1 and FOXO3 both associated with CTNNB1 in untreated and NED cultures of Camel Blue and STE. The degree of this interaction was decreased in ED of Camel Blue and STE (Fig 6.4A). However, CTNNB1 protein levels were not altered due to HRTP exposure, thus suggesting that the diminished interaction between FOXO and CTNNB1 is driven by the diminished nuclear availability of FOXO. Generally, this finding may also point to an inability of additional proteins to shuttle into the nucleus.
Figure 6.4. HRTP-mediated nuclear exclusion of FOXO. A-C) Western blot analysis of cytoplasmic (A), whole cell (B) and nuclear (C) protein fractions. Cytoplasmic and whole cell lysates do not show reduced FOXO protein in Camel Blue and STE effective doses (ED), while nuclear FOXO localization was decreased in Camel Blue and STE treated cells on day 7. D) Camel Blue and STE decrease CTNNB1/FOXO interaction. Nuclear protein extracts were immunoprecipitated with CTNNB1 and Western blots performed. Densitometry suggests decreased presence of CTNNB1/FOXO complex in ED. CTNNB1, beta-catenin; STE, Camel Snus tobacco extract.
shRNA-mediated knockdown of FOXO mimics exposure-associated inhibition of osteogenesis

To causally determine the link between FOXO availability and early commitment of cells that may continue through differentiation to give rise to osteoblasts, we examined the effect of an shRNA-mediated FOXO knockdown on osteogenic differentiation. FOXO hairpin sequences were cloned into the pBK-Flex shRNA vector and human ESCs were then transfected with the resulting shFOXO1 or shFOXO3 plasmids to create stable cell

![Image](image_url)

**Figure 6.5.** Activation of FOXO shRNA reduces FOXO protein levels. (A) Dot blots were performed on whole cell lysates after the addition of Cre recombinase to H9 hESCs stably transfected with a hairpin against the respective FOXO as indicated. 2 µL dots of lysates were probed with primary antibodies. shRNA-mediated knockdown of FoxO (orange boxes) was determined to select clones for future experiments. (B) shFOXO clones were osteogenically differentiated. Cre recombinase was administered on day 5 and cells were fixed on day 7. Clones showed a reduction in FoxO nuclear localization and overall fluorescence intensity compared to the Mock control. Scale bar = 100 µm.
lines. The stable clones were then taken through an osteogenic differentiation and on day 5 of differentiation, Cre was added to induce knockdown. Knockdown was confirmed with a dot blot analysis (Fig. 6.5A). On day 7, immunocytochemistry analysis also confirmed nuclear absence of the FOXOs (Fig. 6.5B). On day 20, a calcification analysis showed reduced calcium content in all but one of the shRNA-FOXO clones (Fig 6.6 A,B). In this, the FOXO knockdown mimicked HRTP-mediated inhibition of osteogenesis. Together, these data suggest that HRTP exposure negatively effects FOXO expression, which in turn can lead to decreased osteoblast differentiation.

![Figure 6.6](image_url)

**Figure 6.6.** FOXOs are required for osteogenic differentiation. (A) Calcium quantification in cellular lysates measured by Arsenazo III showed reduced calcium levels compared to mock transfected cells, n=5± SD, *p<0.05 one-way ANOVA vs mock. (B) FOXO1 and FOXO3 knockdown cells exhibited attenuated osteogenic differentiation. The appearance of the black deposits of a calcified extracellular matrix were reduced (brightfield microscopy). Alizarin and von Kossa staining also showed reduced mineralization. Scale bar = 500 µm.

**HRTPs induce unique expression profiles in differentiating osteoblasts**

While FOXO target promoters are well known in association with oxidative stress removal, it is unclear which down-stream transcriptional changes occur together with a reduction in nuclear FOXO during osteogenesis and/or early osteoprogenitor commitment. Hence, HRTP-associated changes in transcriptional regulation were determined globally
using RNA-seq on day 7 differentiated cells. Principal component analysis (PCA) on normalized data showed that triplicates clustered together within their treatment (Fig 6.7A). Differential expression analysis comparing untreated cells to Camel Blue and STE uncovered that 606 and 181 genes were differentially expressed, respectively (Fig 6.7B). Interestingly, this analysis showed that there were more differentially regulated genes in Camel Blue-treated cells versus STE-treated cells. In determining overlapping differentially expressed genes (DEGs) between the two HRTPs, a VENN analysis was conducted. The result showed that Camel Blue had 590 genes differentially expressed STE had 163 unique genes expressed (Appendix 2). There were no common DEGs in the

![Figure 6.7](image.png)

**Figure 6.7.** Differential expression of genes treated with Camel Blue and Camel Snus tobacco extract as identified with RNA-seq. (A) Principle component analysis (PCA) demonstrates clustering of Camel Blue and STE treated replicates on day 7 of differentiation. (B) Total number of genes dysregulated in Camel Blue and STE cells compared to untreated control or Camel Blue compared to STE. (C) Venn diagram of Camel Blue and STE compared to untreated genes differentially regulated from panel B. (D) Venn diagram shows the total number of genes differentially regulated in comparison between untreated to STE and Camel Blue to STE. UNT, untreated; STE, Camel Snus tobacco extract; CB, Camel Blue; NED, non-effective dose; ED, effective dose.
combined up- and downregulated genes between Camel Blue and STE (Fig 6.7C). The HRTPs shared 11 genes downregulated and 1 gene upregulated. In comparison, analysis of Camel Blue to STE revealed 832 genes were differentially expressed between the two treatment groups (Fig 6.7D). PCA and heatmap findings suggested that Camel Blue and STE had their own HRTP-sensitive transcriptome. The top DEGs were determined by Log2FC of ≥1.0 (Fig 6.8; Fig 6.9). In the genes that were differentially expressed between untreated to STE and Camel Blue to STE, there were 61 genes in common (Fig 6.10).
Figure 6.8. Highly differentially expressed genes sensitive to Camel Blue exposure during osteogenic differentiation. Graph of dysregulated genes with ≥1.0-Log2 fold change (FC).
Figure 6.9. STE-responsive differentially expressed genes. Graph of dysregulated genes with $\geq 1.0$-Log$2$ fold change (FC). STE, Camel Snus tobacco extract.
Figure 6.10. Genes downregulated unique to Camel Snus tobacco extract treatment. Graph shows the Log2 fold change (FC) of 61 commonly downregulated genes by STE. Unt, untreated; CB, Camel Blue; STE, Camel Snus tobacco extract.
Gene ontology (GO) analysis of Camel Blue and STE DEGs displayed biological processes with unique networks that are each known to have a role in the pathogenesis of bone defects (Fig 6.11). Significant GO terms of p-values less than 0.05 for Camel Blue were found for downregulated genes associated with cell cycle regulation, interaction of CTNNB1/TCF, transcriptional regulation, and nucleosome assembly (Fig 6.12A). The upregulated genes in Camel Blue were involved with mitochondrial assembly, drug, and response to ROS (Fig 6.12B). STE downregulated genes were specific for developmental pathways such as somitogenesis, mesoderm formation, and bone morphogenesis (Fig 6.12A). GO analysis revealed 3 distinct categories for STE upregulated genes for ECM organization, cell shape regulation, and phagocytosis (Fig 6.12B). Notably, Camel Blue downregulated *IGF1R, EP300, EGFR*, and *PTCH2* genes (Fig 6.8). Downregulation of any of these genes results in a reduction of bone (Linder et al., 2018; Klein et al., 2016; Hu et al., 2015; Xian et al., 2010). Interestingly, *IGF1R, EP300*, and *EGFR* are FOXO targets (Chiu et al., 2016; Guntur and Rosen, 2013; van der Heide and Smidt, 2005). STE had the following downregulated genes: *T, EOMES, CDX1, CDX2, MSX2, CER1*, dysregulation of any of which can result in axial, appendicular, or craniofacial malformations. (Fig 6.9; Pennimpede et al., 2012; Savory et al., 2009; Hart et al., 2002, Zhu et al., 2016; Papaioannou, 2014, Papaioannou, 2014; Satokata et al., 2000). Together the RNA-seq results showed that genes that are regulated early in osteoblast differentiation are indeed sensitive to Camel Blue and STE exposure.
Figure 6.11. Camel Blue and Camel Snus tobacco extract regulate different biological processes in differentiating cells. A) Gene ontology networks of Camel Blue. B) STE gene ontology networks. More biological processes were dysregulated in the Camel Blue treated cells. STE altered pathways involved in early development. STE, Camel Snus tobacco extract; GO, gene ontology.
Discussion

Tobacco use is associated with several adverse health outcomes, including defects in bone development. It is important to understand the specific genes and pathways of bone development that are dysregulated due to tobacco exposure. Other groups have focused on the role of osteoclast activity to lead to a skeletal defect. However, there is a gap in understanding of how altered gene expression during osteoblast differentiation can describe osteogenic inhibition. Here we provide here the first report of the effects of Camel Blue...
and STE on human ESC osteoblast differentiation, identified a potential role of FOXO1/3 including a global gene expression analysis.

FOXOs are crucial in mediating longevity, yet their exact role during embryonic development is not well understood. Other studies have shown that the decrease of FOXO in the adult mouse skeleton will inhibit bone homeostasis (Ambrogini et al., 2010). We expand here on this knowledge of FOXO in bone development. Specifically, we show that FOXOs are present on the first day of hESC differentiation and downregulated further into differentiation. After osteogenic differentiating factors are added to the culture medium, both FOXO1 and FOXO3 are increasingly expressed on day 7 of differentiation, showing that FOXO may have a role in early osteogenesis in a time dependent manner and potentially suggesting that expression of FOXOs may be controlled by the vitamin D3 receptor (as vitamin D3 is one of the three osteogenic supplements).

Further, it appears FOXOs play a specific part in the differentiation of neural-crest osteoblasts. Previously we had shown that our osteogenic induction protocol gives rise osteoblasts from the NC-lineage, with positive identification of NC cells on day 7. When we put the H9 hESCs through a NC-specific differentiation protocol, the cells were positive for NC markers as well as FOXO.

HRTP exposure to osteogenically differentiating cells dose-dependently decreased FOXO1 and FOXO3 mRNA expression. The decrease in FOXO was in agreement with previous findings that osteogenesis decreased with increasing concentrations of Camel Blue and STE (chapter 5). In addition, FOXO transcriptional activity, determined by SOD2 expression, was decreased in our HRTP-treated cells. However, we did not see
misregulation of CAT. This is in line with previous findings from our laboratory, in which chromatin immunoprecipitation studies in murine ESCs could not confidently determine FOXO1 and FOXO3 contribution to CAT expression either (McClelland-Descalzo et al., 2016). While CAT is efficient at reducing hydrogen peroxide, CAT is confined to the peroxisomes and may not be the major contributor of hydrogen peroxide detoxification. RNA-seq analysis uncovered the major hydrogen peroxide detoxifying enzyme, glutathione peroxidase (GPX) was downregulated in HRTP-treated cells. Though not concluded to be regulated by FOXO, the lack of GPX further demonstrates the breakdown in cellular defense mechanisms.

In response to environmental stimuli, FOXO interacts with CTNNB1 to upregulate mechanisms to thwart oxidative stress. However, the complex of these two can prevent CTNNB1 from complexing with LEF/TCF, a crucial interaction for osteogenesis (Iyer et al., 2013). Immunoprecipitation analysis showed a reduced interaction between FOXO and CTNNB1. Further, we found lower nuclear protein levels of FOXO. FOXO regulation is controlled through protein-protein interactions and post-translation modifications (Brown and Webb, 2018). For example, acetylation of FOXO leads to its activation and remain in the nucleus to turn on its target genes. Therefore, it is plausible that HRTPs can either prevent FOXO from shuttling into the nucleus or make it prone to exiting through post-translational modifications (Daitoku, et al., 2011).

Others have shown that the silencing of FOXO decreases osteoblast yield (Iyer, 2013; Ambrogini et al., 2010; Teixeira et al., 2010; Almeida et al., 2007). In these studies, FOXO was deleted in mature osteoblasts or at the adult MSC stage. These data represent
defects in skeletal homeostasis relevant to an adult. In contrast to those prior studies, we removed FOXO at an early stage of development and the results we gathered pertain to the developing fetus. During osteogenic differentiation of the shFOXO1 and shFOXO3 clones, the cultures phenocopied the reduction of osteoblast production as seen with the HRTP treatment. These data support the notion that FOXOs reduces bone formation, however add an earlier level of regulation that was previously unknown. Consequently, the hypothesis that HRTPs might suppress FOXOs yielding osteoblast inhibition upon exposure seems feasible.

RNA-seq results revealed that each Camel Blue and Camel STE had their own distinct effects on osteogenesis. Camel Blue had DEGs that broadly targeted cellular functions such as DNA regulation (nucleosome assembly and histone modification), mitochondrial function, and growth receptors (EGFR and IGF1R). Whereas STE had an overwhelmingly amount of DEGs associated to paraxial mesoderm. Nevertheless, each of these mechanistic pathways is linked to bone development. In the data set, we uncovered FOXO targets or genes associated with FOXO such as, C/EBPα, EP300, EGFR, IGF1R, EOMEs, and NODAL. Specifically, all the downregulated genes resulted in diseases associated with bone development and mineralization determined by GO analysis disease pathway (Fig 6.12C). However, further investigation is required for the confirmation of these RNA-seq results and whether these genes are truly regulated by FOXOs.

In sum, this study found that exposure to harm-reduction tobacco products are detrimental to the developing skeleton. Our results suggest depletion of FOXO1 and FOXO3 mRNA and protein in Camel Blue and STE treated cells. RNA-seq analysis
identified HRTP-specific DEGs that are linked to adverse skeletal development. Ultimately, this study is closing the gap on understanding the underlying molecular mechanisms involved in the pathology of HRTP-induced skeletal defects.
References


42. Zhu J, Kwan KM, Mackem S. Putative oncogene Brachyury (T) is essential to specify cell fate but dispensable for notochord progenitor proliferation and EMT. Proc Natl Acad Sci U S A. 2016;113(14):3820-5.

CONCLUSION

Cigarette smoke, a major environmental toxicant, is a complex mixture of over 7,000 chemicals. Tobacco use is the most preventable cause of death in the United States and is a health risk for many diseases including congenital abnormalities. Pregnant mothers who experience difficulties in quitting smoking, they may turn to harm-reducing tobacco products like “Lights” cigarettes or non-combustible chewing tobacco. While, these harm-reduction tobacco products (HRTPs) may seem to be a safe alternative for the smoker, the unborn child may be susceptible to unwanted effects. Because it unknown how HRTPs can affect the developing skeleton, in this study, we used human embryonic stem cells to uncover the potential adverse effects of HRTP on the developing skeleton.

Human embryonic stem cells (hESCs) are a developmentally powerful tool to understand embryotoxicity. Human ESCs are derived from the inner cell mass of the blastocyst and have the capability to give rise to every cell type in the body, including the bone forming osteoblasts. Because hESCs can recapitulate embryogenesis, hESCs hold great promise in assessing the effects of toxicants on cell differentiation and viability. Our laboratory has uncovered a promising alternative approach to study developmental bone toxicity that can replace in vitro murine-based models and the reliance on in vivo animal models.

In this work, we provide the first evidence that HRTPs may be developmentally toxic to the developing skeleton. We show that H9 hESCs have the ability to be differentiated into osteoblasts. The use of 1,25-dihydroxyvitamin D3 with ascorbic acid and β-glycerophosphate enhances osteogenesis and upregulates bone-specific genes. These
osteoblasts exhibit the hallmark mineralization of the extracellular matrix. In addition, it appears that our differentiation protocol, primarily gives rise to osteoblasts from the neural crest lineage, determined by mRNA upregulation of \textit{PAX7} and \textit{SOX10} and decreased \textit{TBRA}.

Human ESCs are a great model for assessing the embryotoxic potential of a chemical. The zur Nieden lab has been at the forefront of \textit{in vitro} embryotoxicity screening models for skeletal development. We employed our osteogenic differentiation model to assess the effects of HRTP-mediated differentiation inhibition. Further, our aim was to uncover the mechanisms that lead to osteoblast inhibition. Preliminary data from our laboratory demonstrated that when osteogenically differentiating hESCs are exposed to extracts of popular brands of tobacco their differentiation potential is decreased. Specifically, we exposed hESCs to mainstream (MS) smoke, what is actively inhaled by the smoker, sidestream (SS) smoke, which burns off the tip of the cigarette, or and Snus, fine grain chewing tobacco, tobacco extract (STE). We chose the popularly publicized and commonly used brand of Camel tobacco products. Mainstream smoke Camel conventional and Camel ‘Lights’ renamed to Camel Blue, termed for being ultra-filtered and having lower nicotine and lower tar levels, was not cytotoxic to the differentiating cultures. Camel SS, Camel Blue SS, and Camel STE inhibited osteogenesis measured by the reduction in calcium levels. One method tobacco may cause osteoblast inhibition is through reactive oxygen species (ROS) production. Oxidative stress, the imbalance between ROS levels and free scavenging enzymes, are known to damage DNA and protein that can result in apoptosis, aging, and disease. We found that Camel, Camel Blue, and STE generated
excessive levels ROS, O$_2^-$ and H$_2$O$_2$, during early stages of osteogenesis. It appeared that the higher levels of ROS generated by Camel SS smoke yielded a cytotoxic effect. In contrast, Camel Blue and STE generated sub-toxic levels of ROS that allowed for continued cell survival but inhibited osteoblast differentiation. With the high levels of ROS, we found diminished antioxidant activity in the tobacco treated cells, thus leaving the tobacco treated cells with an inability to counteract the ROS, therefore, suggesting that HRTPs, Camel Blue and STE, may be more harmful to the developing skeleton by perturbing differentiation.

Enzyme antioxidant activity is regulated through the transcription factor FOXO. The measured high levels of ROS combined with reduced antioxidant activity suggests transcriptional dysregulation of FOXO. The FOXO family of transcription factors counteracts ROS through upregulation of antioxidants (SOD2 and CATALASE) and promotes osteoblast differentiation and development. HRTP concentration-response curves showed that FOXOs were dose-dependently downregulated. Western blot analysis of nuclear protein of HRTP-treated cells showed diminished levels of FOXO compared to the untreated control. There were no detectable differences of FOXO protein in cytoplasmic fractions and whole cell protein. Beta-catenin, essential for bone formation, is a co-transcriptional activator and is known to interact with FOXOs to upregulate responses to oxidative stress. Immunoprecipitation of HRTP protein lysates showed reduced FOXO/beta-catenin interaction in the nucleus. Further investigation is needed for the shuttling of FOXO, but it is plausible that HRTP exposure is dysregulating cellular shuttling machinery and preventing the translocation of FOXO into the nucleus and, hence,
inhibiting FOXO target genes. Through short hairpin RNA (shRNA) knockdown of FOXO, we found that the transcription factor is necessary during embryonic differentiation. The lack of FOXO reduced osteogenic differentiation and phenocopied HRTP-mediated differentiation defects. Future studies will determine if the knockdown of FOXO during hESC-osteogenic differentiation results in high levels of ROS. FOXOs may have a key role in toxicant-induced skeletal defects.

RNA-sequencing has allowed for the global profiling of all the RNA within a cell. Global profiling of embryonic development can play and instrumental role in identifying aberrant pathways driving embryotoxicity. In this study, RNA-sequencing globally identified HRTP-responsive genes. There were more downregulated genes than upregulated genes, and a number of the identified altered genes are known to play a role in osteogenesis. We uncovered that Camel Blue and STE have different pathways to elicit osteogenic inhibition. Of all the differentially regulated genes identified, only 12 genes were shared between the two HRTPs. Gene ontology further demonstrated that different biological processes affects between the two HRTPs. Interestingly, STE had downregulated genes associated to paraxial mesoderm, known for axial bone development. Preliminary ongoing studies, in our lab have discovered rib malformations in mouse pups exposed to STE in utero. Camel Blue showed downregulation of growth factors receptors crucial to bone development and defects in DNA machinery such as nucleosome assembly. Further studies are needed to confirm the RNA-seq findings but while the end result of osteoblast inhibition is the same between the two HRTPs, the pathway misregulated is not the same.
The overall objective here was to determine transcriptional regulators that are responsive to HRTP exposure that can result in skeletal malformations. FOXOs play a part in multiple cell processes from ROS detoxification, cell proliferation, DNA repair, apoptosis, and having a supportive role in osteoblast differentiation through the expression of FOXO target genes. Upstream effectors of FOXO, Phosphatidylinositol 3-kinases (PI3K)-AKT pathway blocks FOXO target gene expression. Growth factors trigger the PI3K-AKT pathway and allowing for AKT to phosphorylate FOXO proteins at three conserved residues. The AKT-mediated phosphorylation of FOXO results in the nuclear exclusion of FOXO and, therefore, the suppression of FOXO target genes (Martins et al., 2016). Consequently, any compound that can act upon the pathway has the potential to perturb cellular processes and create unwanted effects. Based on findings from FOXO protein, mRNA levels, and RNA-seq data, that upon HRTP exposure FOXO protein levels may have been reduced through AKT-mediated phosphorylation that resulted in the inability to counteract ROS and disrupted osteogenesis. Overall, HRTPs may have caused differential activation of pathways that controlled transcriptional regulators that inhibited skeletal differentiation (Fig 7.1).

We have successfully established an in vitro model to study the effects of tobacco exposure on the developing skeleton using human embryonic stem cells differentiating into osteoblasts. We found that FOXO is required for proper osteogenic development. Importantly, small changes in gene expression over a collective of genes can result in defects in differentiation. Global gene analysis identified HRTP-sensitive genes that led to perturbation of skeletal development. All together, these findings provide much needed
valuable insights in understanding the pathology of skeletal defects that can be used by global initiatives such as the Center for Disease Control and Prevention and the Tobacco-Related Disease Research Program to help lead in the prevention of women smoking while pregnant and smoking around infants and young children.
Figure 7.1. Harm-reduction tobacco products transcriptional regulation during osteogenesis. A schematic of the working hypothesis of HRTP-mediated FOXO regulation. Chemicals that target receptor tyrosine kinases have the potential to control cellular processes (i.e., proliferation, apoptosis, protein synthesis, and metabolism). Specifically, receptor tyrosine kinase stimulation occurs through the interaction with growth factors. The upregulation of FGF suggests the activation of the PI3K-AKT signaling pathway and, consequently, the exclusion of FOXO (dashed line) from the nucleus facilitated by AKT phosphorylation. Furthermore, decreased nuclear FOXO resulted in the cell’s inability to remove intracellular ROS and inhibit osteogenesis. Validation of RTKs targeted by HRTPs to regulate FOXO is necessary in further understanding of skeletal development dysregulation. PI3K, Phosphatidylinositol 3-kinases; RTKs, receptor tyrosine kinases. Types of RTKs: FGFR, fibroblast growth factor receptor; EGFR, epidermal growth factor receptor; IGFIR, insulin-like growth factor 1 receptor; PDGFR, platelet-derived growth factor receptor; and VEGFR, vascular endothelial growth factor receptor.
Chapter 2: Supplemental information

Appendix Figure 1.2.1. Pilot differentiation studies for protocol development. To determine whether VD3 can induce osteogenesis in human PSCs as it does in mouse ESCs [6], dissociated hESC clusters were first forced to aggregate and form embryoid bodies (EBs), an approach commonly used for mouse and non-human primate ESCs [5, 11, 12, 46]. Human ESCs formed EBs when in the maintenance medium, mTeSR (A). However, upon enzymatic dissociation for single cell replating, in DMEM +20% FBS, an essential step during mouse osteogenic induction [47], low cell attachment and survival was noted. (B) Assays for ALP activity and calcium deposition in H9 hESCs differentiated according to the schematic shown in Fig. 2A show that VD3 outperforms DEX, n=5 independent samples ± SD, *p<0.05 Student’s t test. DEX, dexamethasone; VD3, 1α,25 dihydroxy vitamin D3.
Appendix Figure 1.2.2. Kinetic and passage-specific analysis of additional gene expression associated with osteogenesis and early lineage determination. (A-D) mRNA expression of early and late osteogenic genes was determined with qPCR and normalized to GAPDH, n=3±SD. RIV9 and RIV4 hiPSCs failed to show definitive osteogenic patterns. *p<0.05 passage II over I; #p<0.05 passage III over I; Δp<0.05 passage III over II, One-way ANOVA. (E) ALP activity supported findings for ALP mRNA expression, (n=5±SD). *p<0.05 passage II over I; #p<0.05 passage III over I; Δp<0.05 passage III over II, One-way ANOVA. (F) Inorganic phosphate in hPSC cultures inoculated from three different passages (I-III). Data points represent 5 technical replicates for each biological passage shown ± SD. *p<0.05 passage II over I; #p<0.05 passage III over I; Δp<0.05 passage III over II. ALP, alkaline phosphatase; Runx2, Runt-related transcription factor 2; OPN, osteopontin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Appendix Figure 1.2.3. Inter- and intraline variability between different passages of hPSCs used for osteogenic differentiation. Data points were normalized between different passages of hPSC lines and expressed as n-fold over H9 per time point of analysis. N-fold regulations were then analyzed in box plots. This revealed that the passage-to-passage variability between the three tested lines is similar for most endpoints.
Appendix Figure 1.2.4. Early lineage determining genes are differentially expressed in the three hPSC lines. Messenger expression for genes associated with mesoderm, neural crest and neuroepithelium was quantitatively analyzed with qPCR, on n=3 independent samples ± SD from cells harvested during differentiation. *p<0.05 One-way ANOVA over H9. ZIC1 was low in RIV9 cells, but not statistically different than in H9 cells. GBX2 showed a trend similar to the other neural crest genes, but expression in RIV9 was not statistically different than in H9 cells. NTE5 was highly expressed in RIV9 cells. Although selected as it encodes CD73, an antigen often used to isolate mesenchymal stem cells and therefore expected to associate with a cell that specifies into the mesenchymal lineage from the neural crest, its expression in the RIV9 cultures may simply determine the undergoing mesenchymal commitment of mesodermal cells. Similarly, DLX5 and DLX6 were investigated as they represent markers of neural crest osteoprogenitors. However, their high expression in RIV9 cells, which showed little expression of neural crest genes, may be interpreted as marking all osteoprogenitor cells independently of lineage heritage.
Appendix Figure 1.2.5. Correlation between PAX7 and TWIST1 methylation status and osteogenic differentiation yield across hPSC lines. (A) Methylation-specific PCR was performed across multiple different hESC and hiPSC lines. The result indicated varying degrees of methylation at the TWIST1 and PAX7 promoters independently of cell type. (B-E) The linkage between PAX7 and TWIST1 and methylation state was further explored in RIV1 and RIV7 cells, which represent two hiPSC lines with abnormal karyotypes. RIV1 hiPSCs exhibited trisomy 12, which is a common aberration acquired by PSCs in culture [61] associated with a competitive growth advantage. While chromosome 12 codes for multiple pro-neural crest and pro-osteogenic genes, such as SP7, WNT1, WNT10b, IGF1 and VDR, RIV1 cells had incorporated 71 µg of Ca\(^{2+}\) per mg protein by day 20. Although slightly more than the 44 µg in RIV9 cells this was barely similar to the 90 µg measured in H9 cells possibly due to the two promoters showing patterns more similar to RIV9 cells. RIV7 cells showed an abnormal karyotype with trisomy 5 in 35% of the cells and additional isolated occurrence of trisomy 12 and an isochromosome of the short arm of chromosome 3. The PAX7 and TWIST1 methylation patterns in this line were more like the neural crest conducive H9 methylation patterns and thus these cells displayed enhanced calcification. M, methylated; U, unmethylated.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Ta</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>5'-CCACGTCTTACATTTTGGTG-3'</td>
<td>5'-AGACTGGGCTTTGAGTTTGTG-3'</td>
<td>60</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GAGTCAAGCGATTTGGTG-3'</td>
<td>5'-TGTATTTTGGAGGATCTCG-3'</td>
<td>60</td>
</tr>
<tr>
<td>NANOG</td>
<td>5'-GATTGTGGCGCTGAAGAAA-3'</td>
<td>5'-AAGTGTTTGGTGTGCTTTT-3'</td>
<td>60</td>
</tr>
<tr>
<td>OCN</td>
<td>5'-GGCAGCGAGGTAGTGAAGAG-3'</td>
<td>5'-CTGGGGAAGGAGCAGAACTTG-3'</td>
<td>60</td>
</tr>
<tr>
<td>OPN</td>
<td>5'-GAAACGAGTGACCTGGATG-3'</td>
<td>5'-TGAACATGCTGGCTGGAAGG-3'</td>
<td>60</td>
</tr>
<tr>
<td>PAX7</td>
<td>5'-CACTGTGACAGAGCAGCTG-3'</td>
<td>5'-GTCAGGTTCCGCTACACAT-3'</td>
<td>60</td>
</tr>
<tr>
<td>POU5F1</td>
<td>5'-GTATCTCTCGTCCCTTCC-3'</td>
<td>5'-CAAAAAACCTTGCAAGACTTG-3'</td>
<td>60</td>
</tr>
<tr>
<td>SOX1</td>
<td>5'-TTGTGGCATTAGCTTGGGCA-3'</td>
<td>5'-TTTGAATTGGAGGACACTTG-3'</td>
<td>60</td>
</tr>
<tr>
<td>T/BRA</td>
<td>5'-AAGAGGAAATGACAGCTCAA-3'</td>
<td>5'-TACTGCAGGTTGTGAGCAAGG-3'</td>
<td>60</td>
</tr>
<tr>
<td>TWIST1</td>
<td>5'-ACTGGCCGCAAGAACCATA-3'</td>
<td>5'-TGCAATTACATTGGTCCT-3'</td>
<td>60</td>
</tr>
<tr>
<td>RUNX2</td>
<td>5'-TTTGACACTGGGTCTGCTT-3'</td>
<td>5'-TGGCTGACATGAAAGACTG-3'</td>
<td>60</td>
</tr>
<tr>
<td>SATB2</td>
<td>5'-GCAATTTAGCGAGCAGCAAC-3'</td>
<td>5'-GTGGTCGGTGCGAGGTTTT-3'</td>
<td>60</td>
</tr>
<tr>
<td>ZIC1</td>
<td>5'-GTCTCACACGCCATCCAGT-3'</td>
<td>5'-GCGATAAGGACCTTGTTGTGC-3'</td>
<td>60</td>
</tr>
</tbody>
</table>

**Round 1 of TWIST1 Methylation-Specific nested PCR amplification**

| TWIST1 methylated | 5'-CGTATTGGGTGTTGGGGGGGC-3' | 5'-ACCTAACCCGAAGCCGAG-3' | 58 |
| TWIST1 unmethylated | 5'-GGTGTTGGTTGGGG-3' | 5'-ACCTAACCCGAAGCCGAG-3' | 58 |

**Round 2 of TWIST1 Methylation-Specific nested PCR amplification**

| TWIST1 methylated | 5'-GGGCGGTTGTGATGTTTGGT-3' | 5'-CACCCGCTCCTAACCCGAG-3' | 58 |
| TWIST1 unmethylated | 5'-GGTGTTGGTTGGGG-3' | 5'-CACCCGCTCCTAACCCGAG-3' | 58 |

**PAX7 Methylation-Specific PCR**

| PAX7 methylated | 5'-TTTATGAAATAAAAAGGAGTTTGA-3' | 5'-CTAAACGAAAAACAAAATACA-3' | 60 |
| PAX7 unmethylated | 5'-TTTATGAAATAAAAAGGAGTTTGA-3' | 5'-AACAAAAAATACAAAATACAAA-3' | 60 |

**Appendix Table 1.2.1.** Oligonucleotide sequences used in this study. Ta, annealing temperature.
Appendix Figure 1.4.1. Testing of three control chemicals in the hESC-ESTo. (A) Photomicrographs of unstained and stained H9 hESC osteogenic cultures on day 30 of differentiation. Bar = 100 µm. (B- D) Concentration response curves for PenG, 5FU and atRA. (B) mg Ca2+ per mg protein measured in hESC osteogenic cultures after chemical exposure normalized to solvent controls. (C) Cell viability on hESCs measured with MTT assay. (D) Cell viability of exposed human foreskin fibroblasts (HFF). Values are representative of five technical replicates ± SD for each biological replicate, *p<0.05 One-Way ANOVA. hESC, human embryonic stem cell; HFF, human foreskin fibroblast; PenG, penicillin G; 5FU, 5-fluorouracil; atRA, all-trans Retinoic acid.
Appendix Figure 1.4.2. The effects of 13cisRA on somatic cells. (A) Endpoints in the hESC-ESTo: Human fibroblast (HFF) viability determined by MTT. (B) Endpoints in the mESTo: cell viability tested on mouse 3T3 fibroblasts. \( n=5 \pm SD \) for each experimental replicate, \(^*p<0.05\) One-Way ANOVA.
Appendix Figure 1.4.3. TWIST1 mRNA expression as endpoint in the hESC-ESTo. (A) Effects of tested compounds on the expression of the osteogenic progenitor gene TWIST1 on day 10 of differentiation determined with qPCR and normalized to GAPDH, n=3±SD. *p<0.05 One-Way ANOVA over untreated control. (B) Correlation between half-maximal inhibitory doses found for cell viability (IC50) and differentiation (ID50Ca) with human ESCs and iPSCs. H9 hESC, human embryonic stem cell; RIV9 hiPSC, human induced pluripotent stem cell; PenG, penicillin G; SFU, 5-fluorouracil; 13cisRA, 13-cis Retinoic acid; atRA, all-trans Retinoic acid.
## APPENDIX 2

### Chapter 6: List of differentially expressed genes

**Camel Blue UP genes**

<p>| RPL7AP30       | NOP10         | PFDN4       |
| MRPS21         | POLR2H       | RPL34       |
| EIF1B          | MGMT3        | MTX2        |
| RPRM           | MRPL27       | CD9         |
| RNFL81         | TIMMDC1      | PLGRKT      |
| RPI1-452N17.1  | LINC01405    | PGLS        |
| ECI3           | RPS20P10     | RP11-734E19.1 |
| ATP5A1P2       | CTSL         | WFD2C       |
| EDF1           | MRPS33       | RPL24P4     |
| SRP9           | FAUP1        | MFSD3       |
| RPL7AP6        | DGU0K        | TCEAL9      |
| UQCRFS1        | PMVK         | SDF2L1      |
| POLR2K         | INOS0B,WBP1  | DYNLT1      |
| SF5B3          | TPRKB        | PTGES2-AS1  |
| SF5B5          | MRPL12,SLC25A10 | DUSP23   |
| AGPAT2         | BEX3         | S100A6      |
| RPS19P1        | PFN1P1       | HNRNPLP1    |
| SPAG7          | CLPTM1L      | SAT1        |
| RP11-298C3.2   | ZCCHC9       | PRDX5       |
| RPL35P5        | CEBP3G10.1   | ZNF593      |
| RAB13          | CETN3        | RPS12       |
| COX5A          | GAMT         | RBX1        |
| UQCR10         | CIR1         | RPS4Xp11    |
| RG516          | ID1          | C9orf142    |
| RP11-641D5.1   | CCDC37L      | COX7A       |
| PSMB6          | HDHD5        | MT2A        |
| TPM2           | GAR1         | SNRPG       |
| FDX1           | TEMME101     | RPL6P27     |
| LINC00998      | TEMM54       | ARL6p1,OGFD2 |
| BIK            | NUDT1        | RP11-114H7.1 |
| CKS2           | RPP25L       | RWDD1       |
| RPL5P17        | RIDA         | RP1-159A19.3 |
| ISO2C          | C19orf43     | TXN         |
| RP1-278E11.3   | BLOC1S4      | ATP5E       |
| BLVRB          | RPL34P18     | PYCARD      |
| TACSTD2        | FTHIP8       | TACSTD2     |
| RPS7           | RPS27AP16    | ABRACL      |
| METTL26        | NUDFS5       | RPS15       |
| HSD17B10       | TEMM150C     | PGF         |
| NDUFV2P1       | MDK          | CLND2,ETFB  |
| GTF3C6         | NDUFA12      | RPL36AL     |
| PITHD1         | CHCHD2       | C12orf57,RNU7-1 |
| MCRIP1         | CIB1         | RPS20,SNORD54 |
| SOD1           | C20orf27     | CYBA        |
| FTL5P3         | RAC3         | RP3-340B19.2 |
| PSMA7          | SDHAF1       | PIR         |
| ATP5LP2        | HMGB2        | ACC07969.5  |
| NDUFA8         | HSPB1        | ATP5F1      |
| SNORD50,VPS29  | NMRK2        | DRAP1       |
| COX5B          | CCS          | ILF3-AS1    |
| RBMX2          | GPX1         | SSBP1,TAS2R6P |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRPS34</td>
<td>LGALS1</td>
<td>SCGB3A2</td>
</tr>
<tr>
<td>RP1-182O16.1</td>
<td>RPS20P14</td>
<td>PDCD5</td>
</tr>
<tr>
<td>RARRES2</td>
<td>SAC3D1</td>
<td>GAL</td>
</tr>
<tr>
<td>NDUFAB1</td>
<td>RPS21P4</td>
<td>SCAND1</td>
</tr>
<tr>
<td>AP2S1</td>
<td>FAM96A</td>
<td>FIS1</td>
</tr>
<tr>
<td>KCTD14,NDUFC2</td>
<td>RARRES2</td>
<td>RPS7P10</td>
</tr>
<tr>
<td>HEBP1</td>
<td>MAMDC4,PHPT1</td>
<td>COMTD1</td>
</tr>
<tr>
<td>RP11-864N7.2</td>
<td>MRPL54</td>
<td>SFT2D1</td>
</tr>
<tr>
<td>SNRPEP4</td>
<td>LINC01356</td>
<td>TNNT1</td>
</tr>
<tr>
<td>IF6</td>
<td>AURKAIP1</td>
<td>UNCP50</td>
</tr>
<tr>
<td>EEF1B2P2</td>
<td>EEF1B2P2</td>
<td>DNPH1</td>
</tr>
<tr>
<td>SNHG5,SNORD50B</td>
<td>MRPL52</td>
<td>RPS21</td>
</tr>
<tr>
<td>HPN</td>
<td>RP11-234N17.1</td>
<td>ADAM15,DCST1</td>
</tr>
<tr>
<td>H2AFJ</td>
<td>SNRPFP1</td>
<td>DPP7</td>
</tr>
<tr>
<td>COMMD6</td>
<td>EBPL</td>
<td>DPM3</td>
</tr>
<tr>
<td>RPL24P8</td>
<td>MRPL52</td>
<td>NDUFB1</td>
</tr>
<tr>
<td>GADD45GIP1</td>
<td>RPL24P8</td>
<td>SEC61G</td>
</tr>
<tr>
<td>RPS27A</td>
<td>SNRPFP1</td>
<td>RP11-220D10.1</td>
</tr>
<tr>
<td>CCDC167</td>
<td>CCDC167</td>
<td>RPL35AP21</td>
</tr>
<tr>
<td>RPL23AP65</td>
<td>RPL24P8</td>
<td>CCDC34</td>
</tr>
<tr>
<td>METRN</td>
<td>NR2F2</td>
<td>FOXD3-AS1</td>
</tr>
<tr>
<td>DBI</td>
<td>RXFP2</td>
<td>TCF15</td>
</tr>
<tr>
<td>NDUFS7</td>
<td>ALKBH7</td>
<td>DAXX</td>
</tr>
<tr>
<td>RP11-466H18.1</td>
<td>RPL24</td>
<td>LGALS16</td>
</tr>
<tr>
<td>SC22CB-1E7.1</td>
<td>UPLF3B</td>
<td>TEM145</td>
</tr>
<tr>
<td>COP9</td>
<td>RPL22L1</td>
<td>MIB2</td>
</tr>
<tr>
<td>FKBP2</td>
<td>RPS21P4</td>
<td>RPL17P36</td>
</tr>
<tr>
<td>RPL24</td>
<td>LAMTOR2</td>
<td>FBXL15</td>
</tr>
<tr>
<td>UPLF3B</td>
<td>NDUFS6</td>
<td>DPPA5</td>
</tr>
<tr>
<td>RPL22L1</td>
<td>TMEM208</td>
<td>TPGS1</td>
</tr>
<tr>
<td>RPS21P4</td>
<td>SNRPD2</td>
<td>DGCR6</td>
</tr>
<tr>
<td>LAMTOR2</td>
<td>SCGB3A2</td>
<td>GADD45G</td>
</tr>
<tr>
<td>NDUFS6</td>
<td>RPS21P4</td>
<td>IL32,RNU1-125P</td>
</tr>
<tr>
<td>TMEM208</td>
<td>SCGB3A2</td>
<td>HOXB-A5,AS3</td>
</tr>
<tr>
<td>SNRPD2</td>
<td>SCGB3A2</td>
<td>NR2F2-AS1</td>
</tr>
</tbody>
</table>
Camel Blue DOWN genes

SNORD3A, RMRP, 7SK, RNT5L3, HIST1H3A, HIST1H3B, PTCH1, RNU5E-6P, HIST1H1E, HIST1H12C, SCARNA2, HIST1H12E, HIST1H2AI, HIST1H2AH, HIST1H2BM, HIST1H4D, HIST1H2BL, HIST1H2AD,HIST1H3D, HIST1H3A, HIST1H4H, HIST2H2BF, HIST1H2AL,HIST1H2BN, HIST1H2BC, TERC, RCC1,SNHG3,SNORA73, RNU2-2P,WDR74, HIST1H4C, HIST1H2AM,HIST1H3J, RNU12, HIST4H4,RP11-174G6.5, HIST1H3H, HIST1H2AG, HIST1H2BD, CH507-513H4.5, SNHG17,SNORA71, COL15A1, SNORD118,TMEM107, HIST1H2AP54, HIST1H3G, CDKL1, HIST1H4J, HIST2H2BE, MIR4687,STIM1, AC007362.3, TXLNB, RP11-1100L3.4, SLC5A9, HIST1H2AC, RP11-544A12.4, UCAI, USP3-AS1, GRIAI, MIR6516,SCARNA16, CACNA2D4, RP11-310E22.4, STARD13-AS, ZNF66, PRH1,PRR4, LINC00632, AATBC, C10orf90, CCT6P3,RP11-460N20.3, LINC01719, RP11-520B13.8, MIRLET7D,MIRLET7F1, ZNF221, NIP1P3,SMG1P3, IL6STP1, ZNF460,ZNF543, FMN1, IGF1R, MIA40T, MIA40NB, USP37, KCNQ1OT1, TERT, HSPD1, MAPK6PS4, BRWD1, OSBP1L0, MED13L, VEZF1F1, RAPGEP5, MAM12, RNF169, EGFR, BNC2, AC092835.2, FBN2, RP3-425P12.4, ZDHHC20P4, ZNF43, RP3-514P16.1, ATRNL1, BMPR2, PPXFB1P, IF08P1, KCNNH1, OPRD1, PARP14, STXBP5, CDC42BPA, ZNF780A,ZNF780B, TAF1L, ACADM,RABGGTB, MIR5232,TAOK1, ALPK3, RUNX1T1, HIPK3, PEAK1, ASXL2, RPL18A,SNORA68, ATP11A, NOTCH2, WDR82P2, CDON, TNP01P2, ZNF124, TIPI, FGDS, TLIKP1, AC073046.25, TET3, ITG8B, DOCK1, EXPH5, KIAA1549, GIT2, PRICKLE2, SPATAS, PPP1R9A, MSN1P, ZNF469
<table>
<thead>
<tr>
<th>Gene 1</th>
<th>Gene 2</th>
<th>Gene 3</th>
<th>Gene 4</th>
<th>Gene 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTD-2651B20.1</td>
<td>SHF</td>
<td>RP11-3OP6.1</td>
<td>WDFY3</td>
<td>CECE2</td>
</tr>
<tr>
<td>CECER2</td>
<td>WDR58P1</td>
<td>RP1-102E24.6</td>
<td>CLIC4P3</td>
<td>SCNP1</td>
</tr>
<tr>
<td>SCDP1</td>
<td>HNRC6B</td>
<td>RP11-428P16.2</td>
<td>P4HC1P1</td>
<td>MTR</td>
</tr>
<tr>
<td>PPARC1B</td>
<td>BCC3.MIR3191</td>
<td>ZKSCAN8</td>
<td>NHLRC2</td>
<td>RP13-88F20.1</td>
</tr>
<tr>
<td>AGO1</td>
<td>LINC00694</td>
<td>ZNF732</td>
<td>LINC00691.A</td>
<td>KIAA1217</td>
</tr>
<tr>
<td>POLR2A</td>
<td>XYL1T</td>
<td>RREB1</td>
<td>LIFR.MIR350</td>
<td>EIF3LP1</td>
</tr>
<tr>
<td>FREM2</td>
<td>PDE4D</td>
<td>UBN2</td>
<td>EIF4BP7</td>
<td>ANKRD50</td>
</tr>
<tr>
<td>PTPN23</td>
<td>DNAJC6.RNU2-15P</td>
<td>TET2</td>
<td>KIRREL</td>
<td>MAPT1B</td>
</tr>
<tr>
<td>ARHPAP32</td>
<td>EIF4BP3</td>
<td>HNRNPA2</td>
<td>MAPK3A</td>
<td>TBC1D16</td>
</tr>
<tr>
<td>NF2</td>
<td>HIST3H2A</td>
<td>PARP4</td>
<td>ANKRD50</td>
<td>TBC1D16</td>
</tr>
<tr>
<td>FYCO1</td>
<td>HSPA8P8</td>
<td>GABRQ</td>
<td>GABRQ</td>
<td>SCF4</td>
</tr>
<tr>
<td>AC063961.1.KIAA1217</td>
<td>HSPB1</td>
<td>GABRQ</td>
<td>GABRQ</td>
<td>TBC1D16</td>
</tr>
<tr>
<td>LINC00694.ZNF445</td>
<td>AC004893.11,TTRAP</td>
<td>HSPB1</td>
<td>GABRQ</td>
<td>SCF4</td>
</tr>
<tr>
<td>SOGA1</td>
<td>AC063961.1.KIAA1217</td>
<td>HSPB1</td>
<td>GABRQ</td>
<td>SCF4</td>
</tr>
<tr>
<td>CDKL5</td>
<td>SNORA40,SSH1</td>
<td>INT6S6P1</td>
<td>ZBTB40</td>
<td>ZBTB40</td>
</tr>
<tr>
<td>SNORA40,SSH1</td>
<td>INT6S6P1</td>
<td>ZBTB40</td>
<td>ZBTB40</td>
<td>ZBTB40</td>
</tr>
<tr>
<td>ZN7F32</td>
<td>SLX4</td>
<td>PPATP1</td>
<td>KIAA1671</td>
<td>ROCK2</td>
</tr>
<tr>
<td>AC004893.11,TTRAP</td>
<td>SLX4</td>
<td>PPATP1</td>
<td>KIAA1671</td>
<td>ROCK2</td>
</tr>
</tbody>
</table>
Camel Snus Tobacco Extract UP Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDR</td>
<td>11q22.31</td>
</tr>
<tr>
<td>HLTf</td>
<td>1q41.2</td>
</tr>
<tr>
<td>HPN</td>
<td>1p32</td>
</tr>
<tr>
<td>KLF9</td>
<td>5q31.31</td>
</tr>
<tr>
<td>CHP2</td>
<td>1q32.31</td>
</tr>
<tr>
<td>LINC00698</td>
<td>1p36.31</td>
</tr>
<tr>
<td>ACTN3</td>
<td>10q22.31</td>
</tr>
<tr>
<td>RP11-324H9.1</td>
<td>8p11.24</td>
</tr>
<tr>
<td>ADRA2A</td>
<td>2p12</td>
</tr>
<tr>
<td>TXNIP</td>
<td>1q21.32</td>
</tr>
<tr>
<td>ATRX</td>
<td>1q21</td>
</tr>
<tr>
<td>PPH1A4</td>
<td>1p35.3</td>
</tr>
<tr>
<td>MIR922,RUBCN</td>
<td>1p36.31</td>
</tr>
<tr>
<td>MIGA1,RNA5SP21</td>
<td>1p36.32</td>
</tr>
<tr>
<td>PAX3</td>
<td>1q21</td>
</tr>
<tr>
<td>CCDC180,RP11-23J9.4</td>
<td>1q21.32</td>
</tr>
<tr>
<td>ICAM5</td>
<td>1p35.31</td>
</tr>
<tr>
<td>ITGB2</td>
<td>2p16</td>
</tr>
<tr>
<td>C15orf52,RNA5SP392</td>
<td>2p16.12</td>
</tr>
<tr>
<td>PLCZ1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>PRDM7</td>
<td>1q21</td>
</tr>
<tr>
<td>MEPE</td>
<td>2p16</td>
</tr>
<tr>
<td>FAM129C</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-553L6.5,5,5ZBTB20</td>
<td>1p36.31</td>
</tr>
<tr>
<td>DRAIC</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-881I8.2</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RNU6-871P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>LINC00639</td>
<td>1p36.31</td>
</tr>
<tr>
<td>ARL11</td>
<td>1p36.31</td>
</tr>
<tr>
<td>LINC00271</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP5-1182A14.5</td>
<td>1p36.31</td>
</tr>
<tr>
<td>NDUF5P3</td>
<td>1p36.31</td>
</tr>
<tr>
<td>U1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-151I1.2</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RNVU17</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-284L19.1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-472N13.2</td>
<td>1p36.31</td>
</tr>
<tr>
<td>LINC01374</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RNU6-1274P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>EEF1A1P39</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-483F11.7</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP1-305G21.1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-3132.6</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RN7SL519P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-321F8.4</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-124N19.4</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-594C13.2</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RNU1-47P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-457D20.1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>CTD-2520I13.1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>LA16c-349E11.1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RN7SL525P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>Y_RNA</td>
<td>1p36.31</td>
</tr>
<tr>
<td>SFTPB</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RN7SKP185</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-674E16.1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RNU6-922P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP13-1016M1.2</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RN7SL218P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RN7SL802P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>CTD-2201E18.5</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP3-380B8.4</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-151J23.1</td>
<td>1p36.31</td>
</tr>
<tr>
<td>HIST1H2AA</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP5-874C20.7</td>
<td>1p36.31</td>
</tr>
<tr>
<td>DUTP5</td>
<td>1p36.31</td>
</tr>
<tr>
<td>SNORA64</td>
<td>1p36.31</td>
</tr>
<tr>
<td>KB-1090H4.2</td>
<td>1p36.31</td>
</tr>
<tr>
<td>SUMO2P18</td>
<td>1p36.31</td>
</tr>
<tr>
<td>NOP56P2</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RN7SL338P</td>
<td>1p36.31</td>
</tr>
<tr>
<td>RP11-92C4.3</td>
<td>1p36.31</td>
</tr>
</tbody>
</table>
Camel Snus Tobacco Extract DOWN Genes

AC008277.1
CHS07-513H4.5
RP11-415J8.3
RNU5E-1
BCRP3,KB-1995A5.4
SP5
MIR4687,STIM1
DOCK3,MIR4787
MSRB3
CDIP1
RP1-225E12.2,TXLNB
SNHG15,SNORA9
RNU4-2
CER1
CCKBR
RNU4-1
FAM89A,MIR1182
T
MRGPRX1
RNY1
ZRANB2-AS2
HSD3B1
PRR9
RNVU1-7
LINC02095,SOX9-AS1
FYB
EOMES
CDX1
OLFML1
MIXL1
MGLL
RP11-384K6.6
KRT80
BBC3,MIR3191
CDX2
GCM1
HAND1
PLXNA2
KANK4
U1
LRRN2
TMP3
WLS
RNU6ATAC
KCNK13
SNORA11,TRO
VPR1-AS1
VGLL1
DI03
VIT
LYPD1
SP6
CEBPA
CGA
FAM26E
ACADM,SNORD45
ADGRG1
MUC15
PLEKHA6
NODAL
MAP1A
LINC01225,LINC01226
REEP1
STS
FAM43A
TBX3
SMPDL3A
CAPN6
MSX2
CDH5
PDE4D
ANKRD1
ZFHX3
GATA6
GATA2
PGF
RPL5P34
GREM2
IFI6
RHOU
TET2
ZNF703
FTH1P7
HSPB8
CTSV
S100A16
CSRP1
Clor115
CLDN4
KRT19P1
RPL37AP8
H2AFY
KRT8P45
TACSTD2
EFNB2
KRT18P27
RP5-827C21.1
RP2
CDC42EP1
S100A11
RHOB
HMOX1
MYL6P3
PRH1,PRR4,TAS2R12
RP11-794A8.1
RNU5B-1
RP11-525G12.1
HIST1H2BA
RP11-89N17.2