THE INTERACTION OF ANDROGENIC HORMONE AND CRANIOFACIAL VARIATION: RELATIONSHIP BETWEEN EPIGENETICS AND THE ENVIRONMENT ON THE GENOME WITH AN EYE TOWARD NON-SYNDROMIC CRANIOSYNOSTOSIS

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THE INTERACTION OF ANDROGENIC HORMONE AND CRANIOFACIAL VARIATION: RELATIONSHIP BETWEEN EPIGENETICS AND THE ENVIRONMENT ON THE GENOME WITH AN EYE TOWARD NON-SYNDROMIC CRANIOSYNOSTOSIS

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Recent research suggests that diversity in craniofacial morphology is produced by a complex interaction of environmental variables including 1) muscle function, 2) genetic factors related to skull growth, 3) timing and heritability of suture fusion (cessation of growth of the joints connecting the bones of the skull), 4) growth and morphology of the brain, and 5) other non-genetic factors including hormones of the endocrine system. How these factors interact in cranial growth and development is not well understood. This dissertation investigated the influence of androgenic hormone on suture bone biology. Methodology used including in vitro cellular challenges, protein analyses, and in vivo therapies. The work described here utilized a large sample size to establish the role of testosterone as a modulator of bone morphogenetic protein and subsequent effects on osteoblast differentiation. Testosterone increased the effect of BMP on osteoblasts, increasing differentiation. The increased differentiation effect was successfully blocked using flutamide, an androgen receptor blocker. Bone cells harvested from non suture calvaria in craniosynostotic rabbits were most susceptible to flutamide administration. The presence of androgen receptors in cells harvested from the suture and non suture bone of craniosynostotic or wild type rabbits could not be confirmed due to a lack of an effective antibody. In vivo administration of flutamide to the coronal suture of craniosynostotic rabbits resulted in greater growth across the coronal suture. However, no correction of craniofacial growth was observed. These results suggest 1) an alternative pathway for dihydrotestosterone's and testosterone's effect on the suture, similar to the adrenal androgens, via the MAP kinase pathway, 2) lack of an effective delivery system of the flutamide treatment, or 3) that an androgen receptor blocker-based therapy is not effective for delaying the eventual fusion of the coronal suture in this model.

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PREFACE

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1.0 INTRODUCTION

1.1 CRANIOFACIAL VARIATION

Variation in craniofacial morphology (i.e., skull shape) has been studied for many years in fossil and living primates (Enlow and Hans, 1996; Krogman, 1978). In humans, this variation has historically been associated with isolation and adaptation to specific geographic regions (Clark, 1971; Mooney et al., 2002). Recent research suggests that diversity in craniofacial morphology results from an inherent plasticity of craniofacial traits. There exists a complex interaction of environmental variables (muscle function), genetic factors, timing and heritability of suture fusion (cessation of growth of the joints connecting the bones of the skull), growth and morphology of the brain, and other non-genetic factors including hormones that act on these plasticities (Cohen and MacLean, 2000; Coussens et al., 2008; Coussens and van Daal, 2005; Coussens et al., 2007; Enlow and Hans, 1996; Fujita et al., 2006; Fujita et al., 2004; Meindl and Lovejoy, 1985; Mooney and Richtmeier, In Press; Moss and Young, 1960; Opperman, 2000; Ptak and Petronis, 2008; Wang et al., 2006a). To understand the diversity in phenotypic expressions of craniofacial traits, a better understanding of how these factors interact is necessary.

1.1.1 Short History of Research on Phenotype

Anthropology has a long history of studying craniofacial variation, especially skull shape. Historically, these studies provided description of human variation, including the delineation of "races." Variation in craniofacial morphologies results from a complex interaction of influences, including heritability, diet, biomechanics, and the environment. In the past, anthropological studies have had success in the delineation of populations based on craniofacial morphologies due to consistencies in those influences. However, with the current trend of globalization, population admixture does not allow for the same consistencies in results. Thus, a better understanding of how the influences on craniofacial morphologies interact is necessary for better studies of human cranial variations.

Darwin (1874) in the Descent of Man addressed the issues of skull shape. He suggested that the study of head shapes could garner a great amount of information between and within populations. Anthropology has a long history of utilizing craniofacial variations as indicators of population affinities (Enlow and Hans, 1996; Krogman, 1978). For example, early research, such as that by Karl Pearson, proposed concepts such as the coefficient of racial likeness based on cranial measurements, using classifications from "very intricately related" to "very unlikely related" and "slight divergence" to "very widely diverged (Pearson, 1926)." This research continues to the present. Forensic anthropology in particular continues to rely on antiquated stereotypic racial classification. For example: East Asian and American Indian populations being the most brachycranic or broad skulled, White populations being mesocranic, or in between brachycranic and more dolicocranic, long skulls Black populations, with Polynesian populations being variable (Gill, 1998).

While forensics continues to rely on racial classifications, modern multivariate statistical techniques are the choice of many physical anthropologists. These statistical techniques replace earlier racial categorizations, creating affines or population relatedness groups as units of measurement. These units are an effective way to side step the argument concerning the viability of classifications based on the term "race." In fact, some of these resulting cranial shape data correspond well to geographic location when isolated populations are the unit of study. One example for the estimation of regional variation by quantitative phenotypic traits is the R-matrix method. In this technique, genetic distances from a centroid, the average phenotypic measures of all the populations, are determined from phenotypic trait data (Relethford and Blangero, 1990; Relethford and Harpending, 1994; Hanihara and Ishida, 2009, Gonzalez et al., 2002). Research of fossil hominids also utilizes similar techniques to explore regional variations (Anton, 2002; Baab, 2008; Bruner, 2007).

In isolated populations, cranial shape does exhibit great genic and heritability dependent difference, **Figure 1**. For example, Franklin et al. examined both Khosian and Bantu skulls from isolated populations in South Africa, and demonstrated Khosian derived skulls to be much more brachycephalic than Bantu counterparts (Franklin et al., 2007). These same analyses have attempted to address questions of the peopling of the Americas. Controversially, research, has determined that morphologically non-Northern Asians may have participated in early migrations to the New World. Data obtained from analyses of South American populations corroborates the early migration theory. This multiple migration hypothesis competes with the more accepted genic single wave, out of Beringia model (Gonzalez-Jose et al., 2008; Gonzalez-Jose et al., 2005).

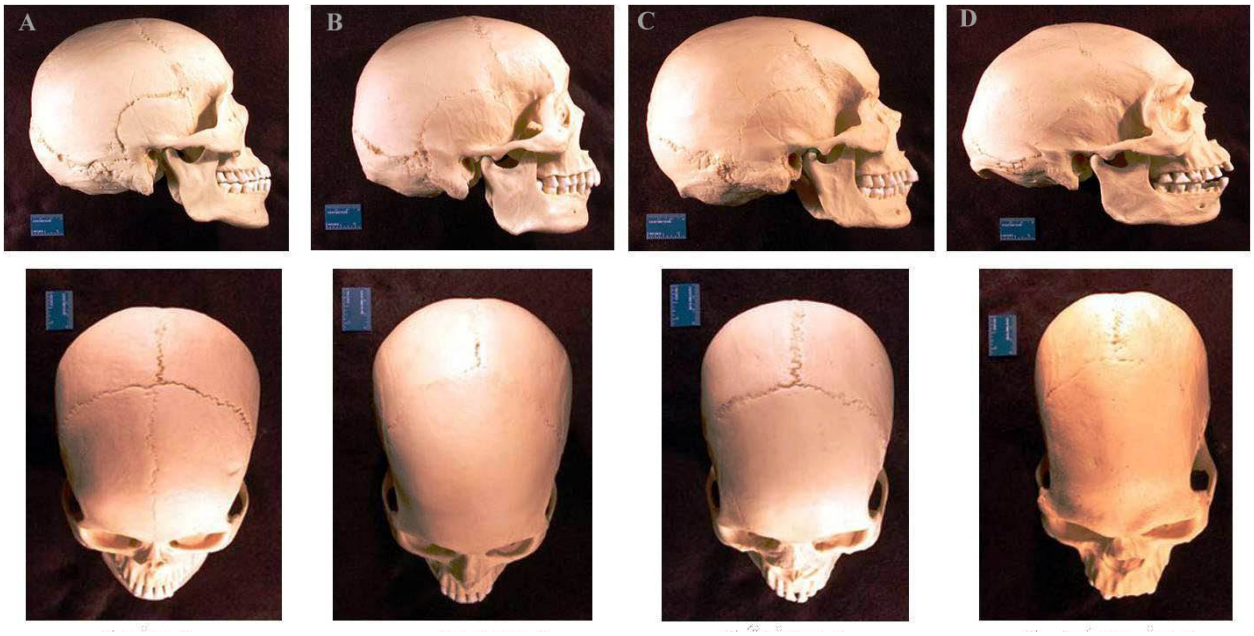

Figure 1: Photographs of Phenotypic Variability in Skull Shape by Geographic Region. Photographs of Phenotypic Variability in Skull Shape by Geographic Region.

A) Asian Derived Skull - Brachycranic Phenotype. **B**) European Derived Skull.- Mesocranic Phenotype. **C**) African Derived Skull – Dolichocranic Phenotype. **D**) Australian Aboriginal Derived – Extreme Dolicocranic Phenotype. Photographs provided by Mark Mooney, PhD., 2009.

An investigation of the effects of climatic signature of cranial shape as well as intra-population variation would allow for a better description of population cranial variability. Although no acceptable explanation has been agreed upon, microevolutionary processes such as drift, gene flow, and directional selection may allow the two seemingly contradictory hypotheses to be reconciled (Gonzalez-Jose et al., 2008; Sardi et al., 2005). On a global scale, Hanihara suggested that African and Australian derived populations shared a more common craniofacial morphology, whereas East Asia and Europeans, and Western Asians and African populations were more similar. This study also highlighted the distinct differences in the craniofacial morphologies of Northern and Southern Asia, pointing to distinct climatic signatures (Hanihara,

1996). In other studies, North American populations appear to exhibit a history of secular trend in cranial shape. Since 1840, these crania exhibit a higher vaulted cranium, most likely reflecting dietary and environmental change with respect to stock European populations (Jantz and Meadows Jantz, 2000).

In addition to genic factors, populations are under similar environmental influences than can increase the likelihood of intra-population similarities in craniofacial variation. For example, diet is a known influence contributing to craniofacial variation. The transition from the dolicocephalic form in pre-Neolithic technological hunter gatherer societies to a more brachycephalic phenotype is often attributed to a softer carbohydrate heavy diet (Stynder et al., 2007). However, Gonzalez and colleagues failed to detect this trend in 18 populations of skulls from known economic strategies in South America (Gonzalez-Jose et al., 2005). Altitude and temperature stresses also influence craniofacial variation. For example, larger crania have a positive association with human populations from colder climates (Roseman and Weaver, 2004). However, this strength of this association has recently been called into question (Harvati and Weaver, 2006). Through experimental animal modeling, Riesenfeld demonstrated a similar degree of brachycephalization in heat raised and starvation rats. Thus, heat induced changes were the result of body weight loss. Cold weather brachycephalization seemed to result from the influence of body weight loss and facial shortening. An increase in cranial height was also associated with a response to cold weather (Riesenfeld, 1973).

Similarity in biomechanical forces can also increase intra-population similarity in cranial form (Vioarsdottir et al., 2002). In *Homo sapiens,* early brain growth is important in determining the size and shape of the cranium (Enlow and Hans, 1996; Mooney et al., 2002; Moss and Salentijn, 1969; Moss and Young, 1960; Richtsmeier et al., 2006; Weidenreich, 1941).

Moss and Young address mechanical forces that affect craniofacial development, in their description of the functional capsular matrix of the neurocranium. These authors suggest that the brain encapsulated by the dura mater creates a system of forces produced by growth of the brain, placing pressure against this neurocranial capsule, the dura and skull tissues surrounding the brain, causing growth (Moss, 1969; Moss and Salentijn, 1969; Moss and Young, 1960). Research also suggests the shape of the basicranium, specifically cranial base flexion, to affect the final craniofacial form. (Lieberman et al., 2000). Alterations in cranial form can result from other mechanical influences. Artificial cranial deformation is one such influence that affects the mass of the underlying brain as well. Other severe aberrations to neural expansion also affect the shape of the neurocranium, such as hydrocephalus, anencephaly, and macrocephaly (Aldridge et al., 2005a; Aldridge et al., 2005b).

Distinct phenotypes of the craniofacial skeleton can result from early aberration of cranial suture growth. For example, premature fusion of the sagittal suture prior to the completion of brain growth results in scaphocephaly. In contrast, brachycephaly results from early coronal synostosis (Cohen and MacLean, 2000; Enlow and Hans, 1996). However, in normal ontogeny there is a period of stasis following the cessation of neurocranial expansion that precedes cranial suture remodeling and fusion (Cohen and MacLean, 2000; Meindl and Lovejoy, 1985). It has been demonstrated that the pattern of cranial suture fusion later in ontogeny (>25 years of age) is independent of cranial shape (Cray et al., 2009). Research on a group from the Aleutians Islands investigated the role of normal ontogenetic suture fusion with respect to cranial shape. Results revealed the same patterns of fusion for brachycranic and dolicocranic populations. This suggests fusion pattern is independent of cranial shape in Homo sapiens. These patterns were also found to differ from that reported in the literature (Meindl and Lovejoy, 1985). Thus, fusion patterns

may be population dependent. Further, standardized methodology using suture fusion to determine age-at-death may not be applicable to all populations (Cray et al., 2009).

Another factor that affects cranial variations is muscular loading. An example, the temporalis muscle attachment. Data suggest that dolicocephalics manifest high temporal lines (bony attachment sites) due to a reduced surface area for muscle attachment. Animal modeling has demonstrated that removal of the temporalis and masseter muscles limits growth in the length of the skull and other postcranial dimensions. This removal of biomechanical forces, however, does not result in a difference in overall brain size. In addition, supplementing the diet can mediate the limitations to growth in the anterior-posterior dimension. This further suggests nutrition has an effect on cranial form. Additionally, brain size was relatively unaffected by surgical manipulation or differential diet conditions (Riesenfeld, 1967).

Increased musculature differentially affects the morphology of craniofacial skeleton. Hypermasticatory influences on the human cranium are reported to result in a higher vaulted skull (Kean and Houghton, 1982). Vecchione et al. (2007) reported on the craniofacial traits of a hypermuscular mouse model. These mice had significantly shorter cranial vault length, a brachycephalic phenotype, shorter maxillary length, and a longer mandibular body length compared to normal controls. Byron et al. (2008) demonstrated that this same hypermuscular mouse model possess an anterosuperiorly and dorsoventrally compressed temporal bone as part of the brachycephalic phenotype, resulting from the enlarged temporal muscles (Byron et al., 2008). Vecchione et al. (In Press 2009), using this same model also found significantly shorter craniofacial lengths in the knockout model, which suggested that muscle function plays a significant role in the ontogeny of craniofacial growth. It was however noted that early changes

in cellular growth and differentiation may have an equal impact on final form (Vecchione et al., In Review).

1.1.2 Cell and Molecular Factors

Craniofacial development and variation is influenced by intrinsic genetic factors, epigenetic factors (both local and general), as well as environmental influences (Van Limborgh, 1982). A molecular and morphological data analysis of populations suggests the basicranium, temporal bone, upper face, and entire cranium are the most reliable markers of heritability in the craniofacial skeleton. The least reliable indicators were the mandible, upper jaw, and cranial vault (Smith, 2009).

A recent study utilizing DNA samples from Caucasians, Asians, Australian Aborigines, African Americans, and Indians for linkage analysis, has begun to described the genetic variation of craniofacial morphology. These authors utilized 30 blood samples samples for haplotype and 100 others genotyped for the haplotype tag single nucleotide polymorphisms, htSNPs (the limited number of haplotypes in a block), phenotypic polymorphism screenings. This polymorphism screening data was compared to the associated craniofacial analysis (head shape). The minimal informative subsets of single nucleotide polymorhisms, SNPs, (stable base pair variations observed in one region across a population) associated were then compared to the htSNPs. Their results showed *TWIST1* gene to contain no non-pathological SNPs and no polymorphism for craniofacial variation. Thus, the *TWIST1* gene is a highly conserved. In contrast, these authors were successful in identifying 17 SNPs, 6 of which were novel for the *FGFR1* gene. For example, the htSNP g.8592931G to C was found to have a negative correlation

with the cephalic index for all populations. Larger correlations for cephalic index were found in Asians and females. This study suggests that in non-pathological populations, single nucleotide polymorphisms (SNPs) on the *FGFR1* gene are associated with normal craniofacial variation (Coussens and van Daal, 2005).

Factors at the epigenetic level also influence the developing craniofacial skeleton. These epigenetic factors include, local factors, e.g. cytokines (cell signaling molecules), growth factor (substance capable of stimulating cell growth), and prostaglandins (lipid compound), (Downey and Siegel, 2006; McCarthy et al., 2000). Systemic influences also affect craniofacial development and variation. These factors include circulating hormones including parathyroid hormones, thyroid hormones (Bradley et al., 1999; Gamborino et al., 2001; Kornreich et al., 2002; McAlarney et al., 2001) and sex steroids (Abu et al., 1997; Fujita et al., 2006; Fujita et al., 2004; Lin et al., 2004; Lin et al., 2007), including androgens. Epigenetic research is now being conducted to determine influences at the cellular level including effects on DNA methylation, phosphorylation, and histone modification. As this research progresses, a greater understanding of the genomic epigenetic interaction will be achieved. This knowledge base should in turn allow a better understanding of resulting craniofacial phenotypes (Ptak and Petronis, 2008).

The interaction between genome-epigenetic and the environment has been studied for craniofacial development. Most of this research has been conducted on the anomalous development of orofacial clefting. The multifactorial threshold model for cleft lip and palate suggests clefting anomalies result from exogenous and endogenous factors, a gene-environment interactions (Etheredge et al., 2005; Jugessur et al., 2003; Krost and Schubert, 2006; Murray, 2002). The gene-environment model for non-syndromic orofacial clefting proposes that if a genetic predisposition for an anomaly is coupled with a environmental factor that can exacerbate

this already existing predisposition, more severe phenotypes will result (Murray, 2002). The identified orofacial clefting candidate genes include TGFA, TGFβ3, and MSX1. A large amount of orofacial clefting research has elucidated the effects of maternal smoking (Jugessur et al., 2003; Murray, 2002; Shi et al., 2007; van Rooij et al., 2002; Zeiger et al., 2005), diet, UV exposure (Krost and Schubert, 2006), and altitude (Otero et al., 2007), on resulting craniofacial anomalies. Sex hormones have also been investigated. Data suggest the environment can influence endogenous hormone levels. Further, hormone can also be absorbed via exposure from the environment causing teratogenic effects. (Herbst, 1973; Limbird and Taylor, 1998; Molsted et al., 1997). As research progresses a better understanding of the gene-environment interaction should aid in the diagnosis and management of craniofacial anomalies (Murray, 2002).

1.2 SUTURE BIOLOGY

Cranial sutures are defined as the fibrous tissue joint separating the adjacent margins of the intramembranously derived bones of the skull (Morriss-Kay and Wilkie, 2005). Sutures permit minor movement to take place between bones. They provide four functions: 1. passage through birth canal, 2. shock absorption, 3. brain growth, and 4. prevent of cranial bone separation (Cohen, 2005); (Enlow and Hans, 1996; Hall, 2005; Moore and Persaud, 2007; Morriss-Kay and Wilkie, 2005; Sperber, 2001).

Cranial vault bones are formed from neural crest and mesodermal tissue. The development of these bones proceeds by intramembranous ossification within a layer of mesenchyme, located between the dermal mesenchyme and the meninges. In contrast, cartilages derived from the occipital

somites ossify to form the supraoccipital bone. The supraoccipital bone eventually fuses with the membranous interparietal bones to complete the skull posteriorly. The cranial region of mammalian embryos contains the first four somites and all structures rostral to them. The trigeminal crest is the only region rostral to the somites that contributes to skull development. The trigeminal crest also contributes to the development of the neural plate region as well as the neural tube. The trigeminal neural crest cells maintain a separation from the adjacent mesodermal cranial mesenchyme cells during early development. *Hox* gene expression also plays a major role in early craniofacial patterning. *Hox* expression is inhibitory to the development of the neural crest derived craniofacial skeleton. However, *Hox* is absent from the trigeminal crest, allowing for early neural associated development (Enlow and Hans, 1996; Hall, 2005; Moore and Persaud, 2007; Morriss-Kay and Wilkie, 2005; Sperber, 2001)

There is a relationship between the neural crest-mesoderm tissue boundary and the position of sutures in the skull vault. The boundary between the frontonasal population and the adjacent mesodermal mesenchyme defines the coronal and sagittal sutures. These sutures act as the neural crest-mesoderm interfaces. During early development, the sides and roof of the skull arise by membranous ossification. This ossification is characterized by needle-like bone spicules that radiate peripherally. Presumptive sutures and fontanels appear At the margins of the frontal, parietal, and occipital bones, during this early membranous ossification. The frontal bone, facial sutures, metopic suture, and coronal suture are of neural crest origin. In contrast, the parietal bone, occipital bone, sagittal suture, and lambdoid suture are of mesodermal origin.

The rostral neural crest cells appear to precipitate the signaling required for sutural growth. Sutures develop by a wedge-shaped proliferation of cells at the periphery of the extending bone fields, termed the osteogenic fronts. The osteogenic fronts appear to govern morphogenetic determination of sutural architecture. For example, unequal biomechanical forces result in overlapping or beveled sutures. In contrast, equal biomechanical forces result in end-to-end sutures

that approximate each other in the same plane. Formations of the sagittal, metopic, and lambdoid occur by narrowing of membranous gaps between bones that were previously widely separated. The coronal by contrast overlaps the frontal bone (Morriss-Kay and Wilkie, 2005).

There are three defined systems of sutures for the neurocranium. The coronal ring consists of the continuous coronal-sphenofrontal-sphenoethmoidal sutures and separates the middle from the frontal cranial segment. The lambdoid system separates the middle cranial segment from the occipital bone. Finally, the sagittal suture is continuous with the metopic suture making up the sagittal system (Enlow and Hans, 1996; Hall, 2005; Moore and Persaud, 2007; Morriss-Kay and Wilkie, 2005; Sperber, 2001).

Some genic factors are of importance in the growth and maintenance of cranial sutures. Efnb1 plays a role in formation of the neural crest-mesoderm tissue boundary that forms the coronal suture. The neural crest-mesodermal boundary leads to the initiation of the FGF-FGFr signaling system, which is associated with osteogenic growth and differentiation at the sutural margins (Coussens et al., 2007; Morriss-Kay and Wilkie, 2005; Passos-Bueno et al., 2008). In sutures, FGFs secreted by osteoblasts at the differentiated edge of the bones, activate receptors involved in both osteoprogenitor cell proliferation and the conversion of these cells into differentiated osteoblasts. Once the FGFr signaling system is established in sutures, long term skull growth depends on the maintenance and balance between the differentiation of new bone and proliferation of the osteoprogenitor cell population as a reservoir of potential new osteoblasts (Cohen, 2005; Nie et al., 2006).

Other genetic factors may be important in cranial suture morphogenesis. TWIST1 is expressed in the sutural mesenchyme between proliferating osteoblasts of the frontal and parietal bone edges-separating two bone forming tissues and initiating transcription of Fgfr2. MSX2 and TWIST1 act cooperatively, but in parallel pathways, to control the proliferation and differentiation of

the neural crest derived mesenchyme that forms frontal bones. TWIST1 and FGFr signaling are a fundamental component of the initiation and maintenance of sutural growth (Coussens et al., 2007; Morriss-Kay and Wilkie, 2005; Passos-Bueno et al., 2008). BMP (Bone Morphogenetic Proteins), a secreted diffusible protein encoded by a member of the *TGF*-β family (Transforming Growth Factor, protein family) may be involved in regulating the balance between undifferentiated states of osteogenic cells. Research suggests Sonic Hedgehog (*Shh*) is expressed in the sagittal suture, and may be involved in regulating cranial suture development and intramembranous bone formation (Kim et al., 1998; Opperman, 2000).

Early in ontogeny the cranial sutures are especially affected by the expanding brain, and the neurocapsular matrix as a whole (Moss and Salentijn, 1969). The underlying dura appears to provide signals for cell proliferation and synthesis, and may harbor the growth factor implicated in suture patency and fusion (Mooney et al., 2001; Opperman et al., 1998). Cranial adjustment to the expanding brain takes place by bone deposition at the sutural margins, while the sutures proper remain patent. Cranial growth proceeds perpendicular to each of the major sutures. There is a period of stasis following the cessation of neurocranial expansion and growth, which precedes cranial suture remodeling and fusion (Cohen and MacLean, 2000; Meindl and Lovejoy, 1985). It has been observed that the expanding brain and its quasi-static strain or extreme biomechanical tensile forces (i.e., such as untreated hydrocephalus or distraction osteogenesis) can in some extreme cases affect calvarial suture morphology. This effect is evidenced by compensatory sutural interdigitation (Mooney and Richtmeier, In Press). However, neurocranial expansion is not always temporally related to human suture activity and thus, unlikely to influence eventual suture morphology and fusion (Cohen and MacLean, 2000; Meindl and Lovejoy, 1985).

Functioning sutures are the sites of continuous bone deposition and resorption. Initially, sutures are straight edges of bone separated by connective tissue. Gradually, interdigitation develops and become more prominent with time. For interdigitation to form, develop, and interlock, the distribution of osteoblasts along sutural bone must be uneven with clumps of osteoblasts at the tip of each interdigitation. Sutural interdigitation may permit adjustive movements and/or stress reductions. Many interdigitated sutures exhibit complex and variable patterns. This relationship depends on the presence, absence, or combination of tensile, compressive, and shearing forces. In addition, these variations occur depending on whether these forces affect sutural bone, sutural connective tissue, or both. In general, the longer a suture remains patent before osseous obliteration, the more interdigitated it will become (Cohen and MacLean, 2000; Enlow and Hans, 1996; Hall, 2005; Krogman, 1978; Mooney and Richtmeier, In Press; Morriss-Kay and Wilkie, 2005).

Cyclic strains resulting from masticatory forces may have a high degree of influence on later ectocranial suture morphology and fusion (Byron, 2006; Byron et al., 2004; Byron et al., 2006; Herring, 2008). Data from animal models have shown that bone growth and remodeling at the osteogenic fronts of the sutures is compensatory and occurs in response to tensile and compressive forces (Byron, 2006; Byron et al., 2004; Byron et al., 2006; Herring, 2008). The mechanical properties of sutures differ by loading, but all present morphologically with increased interdigitation, with increased function which allows for greater elasticity. Experimental evidence suggests sutures under compressive forces result in more interdigitation than sutures under tension (Fong et al., 2003; Herring, 2008; Wu et al., 2007). At the cellular level in general, sutural strain magnitudes have been shown to be small, increasing with age and bone deposition rate which is consistent with a quasistatic strain model influencing osteoblastic

activity at the sutural fronts (Henderson et al., 2004). Sutures adapt to strains through compensatory growth at the sutural edge occurring in response to forces that separate the bones. Data from a hypermuscular murine GDF-8 (Myostatin) knockout model has shown a positive functional relationship between masticatory muscle size and sutural complexity. These mice exhibited increased sutural interdigitation and alterations in craniofacial morphology and mandible shape compared to wild-type control mice (Byron et al., 2004; Byron et al., 2006; Vecchione et al., 2007; Vecchione et al., In Review). Data from this same murine model also suggest that the cranial capacities are unaffected (Cray et al., 2008).

The cause of suture closure is unclear, although there may be one or more mechanisms at work. Examples of such factors are vascular, hormonal, genetic, mechanical, or local factors (Cohen and MacLean, 2000). Some evidence also points to sutural loading as a causative mechanisms for sutural fusion, as it appears new bone is produced at the sutures in response to external stimuli (Herring and Teng, 2000). Age related changes in perisutural growth factor concentration gradients especially members of the Transforming Growth Factor-Beta (Tgf-β) family may also contribute to calvarial suture fusion. The Tgf-βs interacting with many other growth factors and genes (i.e. FGFs, MSX) in the sutural ligament and target osteoprogenitor cells at the sutural fronts causing them to produce excess collagen and bone resulting in fusion (Opperman and Ogle, 2002; Poisson et al., 2004; Rawlins and Opperman, 2008) **Figure 2**.

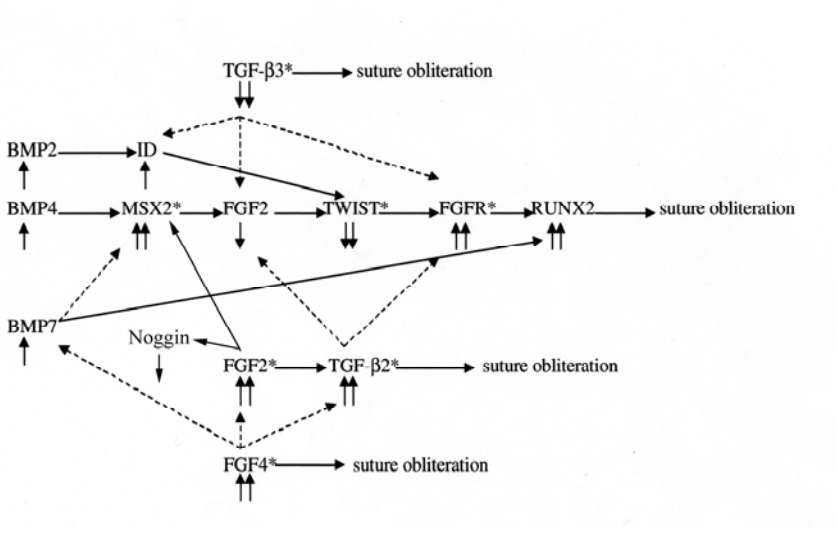

Figure 2: Suggested Interactions Between Genes, Growth Factors, and Suture Morphology

Modified from Opperman and Ogle, 2002

However, there is data on the ontogeny of suture fusion. Initial suture obliteration seems to follow a pattern of slender bony spicules extending from the sutural margins, bridging the sutural gap either partially or completely. The initial bony bridging can develop from either the inner or the outer table in the frontal and lambdoid sutures. In the sagittal suture for example, suture fusion initiation can take place anywhere along its entire length. There does not seem to be any predilection for any area as earliest to fuse. Fusion may also begin on either the endocranial or the ectocranial surface, although it initiates more frequently endocranially. Histological data demonstrates collagenous fibers in the suture have a random organization prior to the onset of fusion. Contrastingly, during osseous fusion, connective tissue cells and fibers decrease in concentration in the sutural area creating an organized trabecular orientation. At this time, collagen fibers increase in tensile strength and decrease in extensibility, creating an organized orthogonal collagen lattice immediately preceding and during fusion, suggesting

suture fusion may be adaptive to dampen strains across the sutures (Anderson et al., 2006; Wang et al., 2006b; Warren et al., 2008), **Figure 3**.

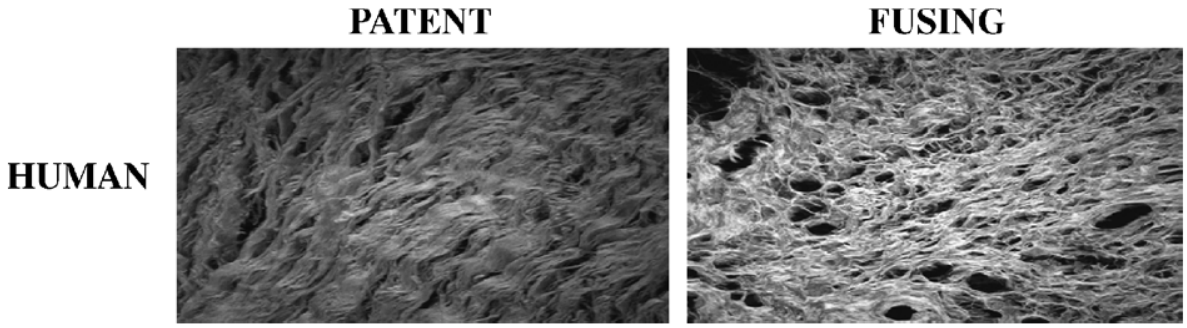

Figure 3: Collagen Fiber Orientation in Patent and Fusing Sutures.

Note the orthogonally oriented collagen lattice in the fusing suture.

Modified from Warren et al., 2008

1.3 CRANIOSYNOSTOSIS

Craniosynostosis is the premature fusion of one or more cranial sutures, the fibrous joints of the skull. If this synostosis happens early enough in the natural history of the animal, it can lead to alterations in skull shape, **Figure 4**, reduced cranial growth, increased intracranial pressure, impaired blood flow, vision and hearing, as well as mental retardation. Craniosynostosis results from an overgrowth of bone at the osteogenic fronts of the affected suture. Distortion of skull shape by mechanical factors, intrauterine pressure, head binding, etc., are not classified as craniosynostosis unless an affected suture is prematurely fused. 8% incidence has been suggested as familial, syndromic and nonsyndromic (Bonaventure and El Ghozzi, 2003; Moore and Persaud 2008; Morris-Kay and Wilkie, 2005; Sperber 2001; (Cohen and MacLean, 2000).

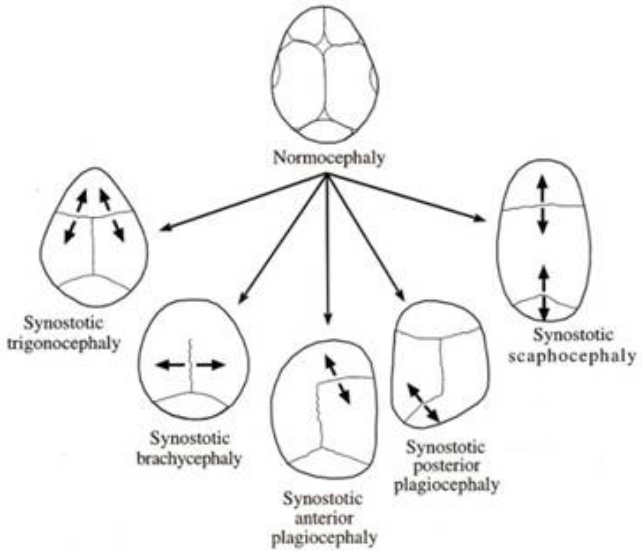

Figure 4: Growth resulting from premature fusion of one or more of the cranial vault sutures

Modified from Cohen and Maclean, 2000

There is some understanding of the genic causes of craniosynostosis in humans. FGFR1-4, belonging to a family of tyrosine kinase receptors, are the most common genes implicated in syndromic craniosynostosis, and the best described. These FGFRs exhibit a common organization, including an immunoglobulin (Ig)-like binding domain, a transmembrane domain and two intracellular tyrosine kinase subdomains (TK1 and TK2). The binding of FGF to FGFR in association with heparin sulphate proteoglycan (HSPG) induced receptor dimerisation at the cell surface and autophosphorylation that triggers phosphorylation downstream signaling proteins (Bonaventure and El Ghouzzi, 2003; Nie et al., 2006). Mutations in Fgfr1-3 are identified causes of syndromic craniosynostosis. For example, Crouzon and Pfeiffer mutations are located in Fgfr2. These mutations occur overwhelmingly in two exons that encode the IgIII a/c domain of the protein. Mutations to the Fgfr2 IgII-IgIII linker region also result in Apert Syndrome. These syndromes present with variable phenotypes, **Figure 5**. However, the phenotypic differences resulting from the three equivalent

mutations do not relate directly to different functions of the genes, they function interactively and loss or gain of function mutations in one gene that affect the function of the protein may have secondary effects on one or both of the Fgfrs.

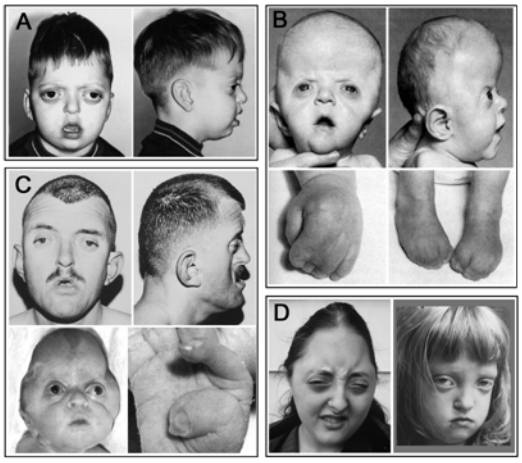

Figure 5: Craniosynostosis Resulting from FGFr Mutations.

A) Crouzon Syndrome. **B**) Apert Syndrome. **C**) Pfeiffer Syndrome. **D**) Jackson-Weiss Syndrome.

Modified from Cohen and Maclean, 2000

Apert syndrome, an autosomal dominant FGFr2 mutation (serine 252 to tryptophan or proline 253 to arginine) is the most severe of the craniosynostosis syndromes. At the cellular level, Apert presents with an increase in calvarial cell differentiation, subperiosteal bone matrix, and premature calvarial ossification. In addition, the fibroblast biology of Apert affected cells suggests greater quantities of glycosaminoglycans and a decrease in circulating Interleukin 1 and 6 (IL1 and IL6). Phenotypically brachycephaly (increased head height and a flattened facial region), hand syndactylies, megalencephaly, hypertelorism, shallow orbits, maxillary hypoplasia, cleft palate, soft palate clefting, midline calvarial defect from glabella to the posterior fontanelle, characterize Apert syndrome. There are very often central nervous system disorders including mental retardation, ventriculomegaly, hypoplasia of the corpus collosum, and agenesis of the septum pellucidum. Other less common phenotypic traits are short humeri and spina bifida (Bonaventure and El Ghouzzi, 2003;

Carinci et al., 2005; Cohen and MacLean, 2000; Cohen, 2005; Ibrahimi et al., 2005; Ibrahimi et al., 2001; Jabs, 2002; Mohammadi et al., 2005).

Crouzon Syndrome, an autosomal dominant FGFr2 mutation (with de novo cases reported), affecting cysteine residue between the IG II and IG III domains, results in accelerated osteoblast proliferation and is stimulatory to interleukin I. There is a large amount of variability in phenotype for Crouzon, generally presenting a similar craniofacial phenotype to Apert syndrome including coronal suture synostosis, but adds ocular proptosis, in some cases scaphocephaly and/or trignocephaly, a reduction in length of the anterior cranial base and clivus, and lacks syndactylies. Other less frequent features include a solid cartilaginous trachea, dental arch shortening anteroposteriorly and in width causing dental crowding, hearing problems, lateral palatal swellings, torticollis, increased cranial pressure. Hydrocephalus can also occur due to jugular stenosis and venous obstruction (Bonaventure and El Ghouzzi, 2003; Carinci et al., 2005; Cohen and MacLean, 2000; Cohen, 2005; de Ravel et al., 2005; Glaser et al., 2000; Jabs, 2002).

Pfeiffer Syndrome, is a mostly de novo FGFr2 mutation resulting from alternative slicing of the B exon on the third immunoglobulin domain (serine to cysteine substitution, or a tyrosine to arginine), also can be found on FGFr1. Pfieffer presents with an increase in cell proliferation and accentuated expression of markers of osteoblast differentiation, i.e. RUNX2. Phenotypic features of Pfeiffer include ocular hypertelorism, proptosis, midface hypoplasia, prognathism, high arched or cleft palate, choanal stenosis or atresia, strabismus, broad and medially deviated great toe and thumb, and brachycephaly due to coronal suture synostosis. Pfeiffer syndrome is subdivided into three phenotypes. Type I or classic Pfeiffer is the mildest form that presents without mental delays. Type II Pfeiffer presents with mental delays, extreme proptosis, elbow ankylosis, or synostosis, and a characteristic cloverleaf skull. Type III lacks the cloverleaf skull. It shares the characteristic brachycephaly of Apert and Crouzon, and the ocular proptosis, but lacks syndactylies. It does

however have hand and feet involvement, exhibiting broad and short thumbs and toes as cited above (Bonaventure and El Ghouzzi, 2003; Vogels and Fryns, 2006).

Jackson-Weiss is an extremely rare autosomal dominant FGFr2 mutation (specifically a missense alanine to glycene coding error) which shares the brachycephaly phenotype of the FGFr2 related syndromes. Other co-morbidities include ocular proptosis, mandibular prognathism, normal intellect, but also lacks syndactylies, exhibiting broad great toes with medial deviation and tarsal-metatarsal coalescence which is the diagnostic feature of the disorder, without hand abnormalities (Bonaventure and El Ghouzzi, 2003; Cohen and MacLean, 2000; Cohen, 2005; Jabs, 2002; Vogels and Fryns, 2006).

Muenke syndrome, an autosomal dominant, with de novo cases reported, is a FGFr3 disorder resulting from proline to arginine mutation, in the extracellular ligand-binding domain, between the second and third Immunoglobulin-like loop. Muenke can present with coronal suture synostosis, or a milder unicoronal synostosis. In addition, the phenotype is characterized by with bulging temporal fossae, ptosis, midface hypoplasia, macrocephaly, high arched palate, coned epiphyses, hearing loss with some patients exhibiting mild limb abnormalities including bracydactyly and carpal and tarsal coalition (Bonaventure and El Ghouzzi, 2003; Cohen and MacLean, 2000; Cohen, 2005; Doherty et al., 2007).

Craniosynostosis occurs from other genic causes as well. Heterozygous missense mutations within MSX2 cause Boston type craniosynostosis. MSX2 encodes a homeobox-containing transcription factors and the mutation, which in the homeodomain, acts by stabilizing DNA binding. In contrast, Saethre-Chotzen has a heterozygous loss of function from mutations in TWIST1. TWIST1 encodes a basic helix-loop-helix transcription factor that is essential for mesoderm formation. EFNB1 gene encodes ephrin-B1, a ligand for EphB receptors. Eph-ephrin interactions are mainly mutually repulsive, and play major roles in preventing cell mixing across boundaries in

embryos (Coussens et al., 2007; Kimonis et al., 2007; Morriss-Kay and Wilkie, 2005; Passos-Bueno et al., 2008).

More than 85% of all cases of craniosynostosis are non-syndromic. Underlying genic causes may also cause non-syndromic craniosynostosis by gene interaction, or gene-environmental interactions. Most of these cases go undetected until an associated clinical diagnosis occurs. Increases in intra-cranial pressure, learning disabilities, or strabismus are examples of such cases. Diagnoses are difficult as the primary defect is not associated with a syndrome. The most common non-syndromic, and/or isolated synostosis occurs at the sagittal suture, resulting in dolicocephaly. Sagittal synostosis is more common in males, perhaps as great as 4:1 (Aviv et al., 2002; Boyadjiev, 2007; Cohen and MacLean, 2000). The second most common non-syndromic synostosis occurs at the coronal suture. This pathology can present unilaterally, resulting in plagiocephaly, or bilaterally, resulting in brachycephaly. Unilateral coronal synostosis makes up about 13% of single suture synostosis cases. Coronal synostosis exhibits a 2:1 tendency occurring more commonly in females (Boyadjiev, 2007; Cohen and MacLean, 2000). Metopic synostosis is rarer and may be an autosomal dominant trait. However, severe cases are associated with hindbrain hernia or Chiari I malformation (Boyadjiev, 2007; Cohen and MacLean, 2000; Pouratian et al., 2007). Isolated lambdoid synostosis, unilateral, or bilateral, resulting in plagiocephaly, is the rarest of all non-syndromic synostosis. In about 5% of all non-syndromic synostosis cases, there is multiple suture involvement (Boyadjiev, 2007; Cohen and MacLean, 2000).

It has been suggested these non syndromic cases are most often sporadic in nature (Aviv et al., 2002). Possible causative mechanisms for non-syndromic cases are, genetic or inheritance, intrauterine constraint, twinning, maternal smoking, preterm labor, hormone involvement, and a genetic-environmental interaction. FGFr1, FGFr2, FGFr3, TWIST, and MSX2 are also suggested

to be involved in non-syndromic synostoses. These non-syndromic cases may be examples of a milder form of syndromic pathologies (Boyadjiev, 2007; Cohen and MacLean, 2000; Johnson et al., 2000; Lajeunie et al., 2001; Lajeunie et al., 1995; Lajeunie et al., 1996).

Craniosynostosis affects not only the craniofacial skeleton, but also the underlying dura and brain. Thus, clinical interventions are necessary to alleviate the complications resulting from craniosynostosis, including increased intracranial pressure that in turn may cause impaired cerebral spinal fluid flow and venous drainage, mental retardation and visual disturbances, strabismus, and Chiari malformations. There are other major causes of cranial asymmetry including positional plagiocephaly, or flattening of the posterior portion of the skull due to mechanical influences. However, radical surgical intervention is necessary for craniosynostosis and its associated co-morbidities. A strip craniectomy and or partial to total calvariectomy are the standard surgical intervention for the affected sutures. These procedures involve the excision of the suture and a portion of the bone surrounding the suture. In more severe cases, particularly those involving multiple sutures, cranial reconstruction is necessary. Complications can include infection, encephalocele, hydrocephalus, dura mater compromise, hematoma, and CSF leaks. The risk of each of these complications increase with multiple surgeries (Chatterjee et al., 2009; Cohen and MacLean, 2000; Esparza and Hinojosa, 2008; Esparza et al., 2008; Inagaki et al., 2007; Kelleher et al., 2007; Murray et al., 2007; Ricci et al., 2007). There is a high risk of resynostosis after surgical intervention in craniosynostosis that causes increased intracranial pressure and abnormal growth, necessitating multiple surgeries, increasing morbidity and mortality (Panchal and Uttchin, 2003; Williams et al., 1997). There is currently no standard of care for the prevention of post-operative resynostosis (Cooper et al., 2007; Cooper et al., 2009; Mooney et al., 2007a; Mooney et al., 2007b), however experimental animal modeling has

investigated multiple potential therapies including surgical techniques and protein and molecular tools.

1.4 HOW ANDROGENS EFFECT CRANIOFACIAL GROWTH

1.4.1 Description of the Pathway

Androgens are any steroid that contributed to the development or maintenance of male characteristics. Androgen receptors mediate most effects of androgens. Androgens are both the precursor for male and female sex hormones. **Figure 6** exhibits the androgenic chemical pathway. Enzymes are necessary for lysing and or catalyzing steroids within the pathway for the creation of the steroid downstream. The androgen molecular pathway begins with cholesterol, and is acted upon by cholesterol desmolase and converted or synthesized to pregnenolone, a prohormone (hormone precursor). Metabolism of this prohoromone occurs by 17α hydroxylase, a cytochrome P450 enzyme that will be important for discussion of craniofacial malformations because of aberrations in the steroid pathway. This process adds a hydroxyl group, hydroxylation, to create 17 hydroxypregneolone, which exhibits a peak concentration during puberty and pregnancy. 17α hydroxylase acts upon this prohormone lysing its side chain to create dehydroepiandosterone, or DHEA. DHEA acts through binding to the androgen receptors or through its metabolites downstream in the pathway. This adrenal steroid is a prohormone for the sex steroids. 3β hydroxysteroid dehydrogenase converts DHEA to androstendione, the common precursor for the estrogenic and androgenic steroid pathways. The production of androstendione occurs both adrenally, mediated by adrenocorticotropin (ACTH), and gonadally,

mediated by gonadotropin (luteinizing, LSH, or follicle stimulating, FSH, hormones). Androstendione is critical for the production of leydig and theca folliculi cells as well as spermatogenic tissue and granulose cells in the gonads. Androstendione is also important for the maintenance of sex steroid levels. The aromatase enzyme acts on Androstendione allowing conversion to estrone and subsequently estrone to estrogen. 17-β hydroxysteroid dehydrogenase can also directly act upon Androstendione allowing conversion to testosterone, the principle male hormone, which has importance for male sex organ development, spermatogenesis, and the maintenance of muscle mass. Androgen receptors mediate testosterone activity. Androgen receptors are a nuclear receptor, which upon exposure to testosterone and dihydrotestosterone translocates to the nucleus. Dihydrotestosterone is a more potent androgenic hormone converted from testosterone by the 5α-reductase enzyme. Translocation of the receptor allows for the regulation of androgen related gene expression for the development and maintenance of the male phenotype (Forest, 1997; Hadley, 2005; Herbst, 1973; Lajic et al., 1998; Meehan and Sadar, 2003; Mo et al., 2006; Nimkarn and New, 2007a; Nimkarn and New, 2007b; Roy et al., 1999; Speiser and White, 2003; Witchel, 2007), **FIGURE 6**.

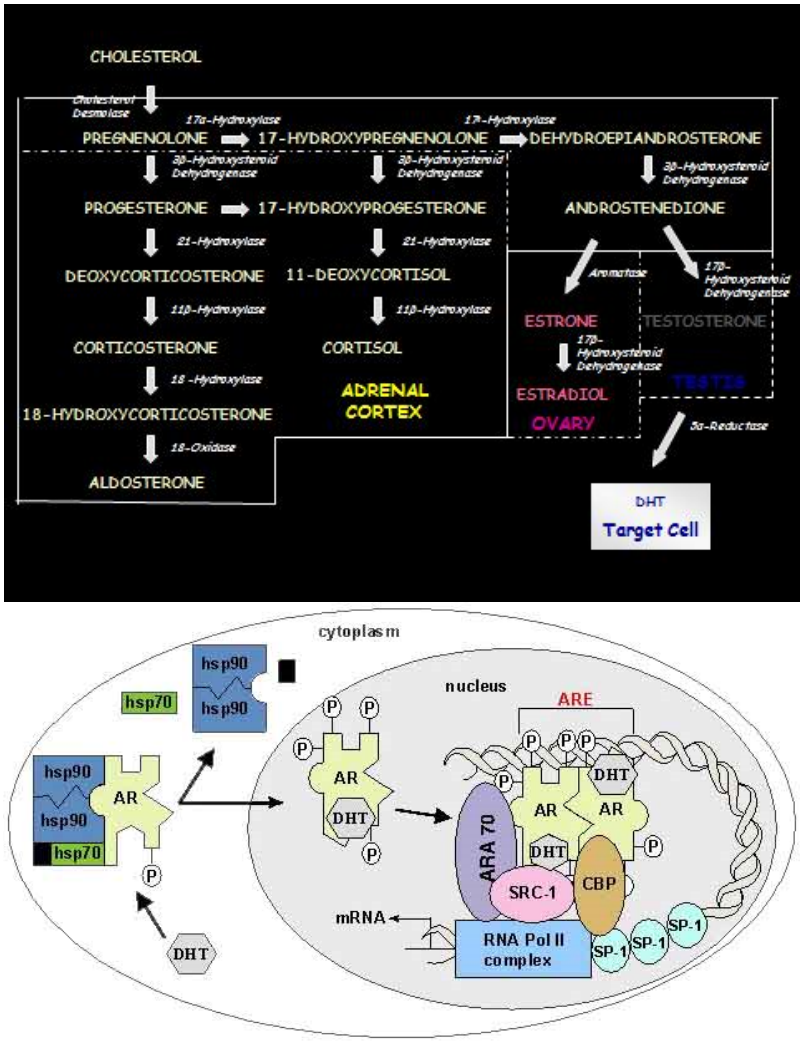

Figure 6: Androgen Pathway and Ligand-dependent Activation of the Androgen Receptor

Androgens such as DHT diffuse through the plasma membrane of the cell and bind to the AR. Upon ligand binding, the AR undergoes conformational changes involving an NH2-/carboxyl-terminal interaction and receptor stabilization. The AR translocates to the nucleus where dimerization and DNA binding to regulatory androgen response elements occurs (114 - 122). AR (androgen receptor); DHT (dihydrotestosterone); CBP (CREB-binding protein); ARE (androgen response element); hsp (heat shock protein); SRC-1 (steroid receptor coactivator 1). Modified from (Meehan and Sadar, 2003; Speiser and White, 2003; Witchel, 2007)

1.4.2 Bone Cell Biology

It is well established that bone responds to hormones (McCarthy et al., 2000). Much research has focused on the effects of estrogen deficiency on bone maintenance and the incidence of osteoporosis (Mansell et al., 2007; Spelsberg et al., 1999). However, short and long-term androgen deficiencies may have detrimental effects on both cortical and cancellous bone. Specifically exposure can lead to stimulation of cancellous and bone turnover in cortical bones. The effects of bone turnover in aging often required clinical prescription of DHT and various adrenal androgens. Experimental research suggests flutamide, an androgen receptor blocker, mediates the positive effects of androgens on bone, via the inhibition of osteoblast proliferation. After a two-year administration period of exogenous androgen, a murine model netted a 45% increase in bone energy absorption capacity, a 39% increase in maximum shear stress, a 23% increase in torsional rigidity, and 15% increase in bending stiffness, a 107% increase in bone elastic modulus, and a 28% increase in compressive stress. These results suggest a great importance for androgen maintenance for the biomechanical properties of long bones (Hofbauer and Khosla, 1999; Hofbauer et al., 1999).

Steroids affect both bone mass and turnover. Rats exposed to exogenous androgen demonstrated increased mineralization, and the prevention of cancellous bone loss. However, the investigators suggest that biomechanical influences may mediate this relationship. Testosterones, like estrogens, have a biphasic relationship with bone maintenances. Although they generally increase osteoblast proliferation, at higher levels or concentrations, they can also inhibit osteoblast proliferation. The same relationship exists for differentiation (Kasra and Grynpas, 1995).

Androgens and androgen receptors influence osteoblast proliferation, differentiation, and apoptosis. The biphasic relationship for these influences has resulted in both stimulation and inhibition of osteoblast proliferation results. However, the adrenal androgen DHEA has been shown

to consistently stimulate proliferation, but with less potency than shown for T and DHT. Androgens also increase apoptosis, which incidentally estrogens prevent. Androgen exposure increases osteoblast differentiation, defined by changes in alkaline phosphates or changes in expression of extracellular matrix proteins, i.e. type I collagen, osteocalcin, osteonectin. In vitro, osteoblasts exposed to androgen increased the proportion of ALP positive cells as compared to control cells in a dose dependent manner. Thus, androgens enhance osteoblast differentiation and may play a role in bone matrix production (Compston, 2001; Wiren and Orwoll, 2002). The adrenal androgens may act through extracellular signal-related kinase or ERKs pathway. However, the more potent testosterone and DHT act by binding to androgen receptors (Compston, 2001; Manolagas et al., 2002; Wiren and Orwoll, 2002).

Androgen receptors influence active bone remodeling. Data from human gingival fibroblasts cells cultured from periodontal patients with actively inflamed tissue demonstrated that the inflamed source had an elevated metabolic response to androgens at baseline and in response to inflammatory stimulus. It has been suggested that this is due to the complex relationship between androgens, specifically DHT studied here, and platelet derived growth factor (PDGF), TGF-β, and IGF-I involved in tissue repair (Kasasa and Soory, 1998a; Kasasa and Soory, 1998b).

It also appears that androgens have a relationship with TGF-βs. Androgens increase TGFβ activity; especially DHT increasing TGFβ2. Research suggests TGFβ may mediate the androgenic effects on osteoblast proliferation, and may determine osteoblast responsiveness. Experimental orchiectomy reduces the amount of TGF-β found in bone. Testosterone replacement experimentally mediates this effect. This suggests reduction in these growth factors, induced by androgen deficiency, influences bone loss associated with low androgen levels (Vanderschueren et al., 2004; Wiren and Orwoll, 2002). Loss of bone mass with age may also be due to loss of adrenal androgens, specifically DHEA, and not the primary sex steroids. Adrenal and the sex steroid androgens have protective

effects for the trabecular bone, acting via the AR and the ER (Compston, 2001; Notelovitz, 2002; Sims et al., 2003; Spelsberg et al., 1999; Vanderschueren et al., 2004; Wiren and Orwoll, 2002).

1.4.3 Craniofacial Growth

Most research concerning excess androgen in animal models have focused on the external genitalia and the inter-sex phenotype (Gray et al., 1994; Wolf et al., 2002), cancer cell research (Broulik and Starka, 1997). However, the deliberate introduction of excess androgen in adult mice has also proven useful in modeling bone loss and osteoporosis (Coxam et al., 1996; Martel et al., 1998; Prakasam et al., 1999; Tivesten et al., 2004). A selection of studies has also characterized some changes to the craniofacial skeleton. Administration of high dosages of nandrolone phenylpropionate, a pharmacological androgen, to rat pups resulted in larger calvarial dimensions, most notably the maxilla-mandibular shape and anteroposterior jaw discrepancy. This suggests over exposure to androgen can affect craniofacial growth (Barrett and Harris, 1993). Research has also been conducted to determine the effects on craniofacial growth after administration of nandrolone phenylpropionate to rats, using cephalometric radiographs at 60 and 120 post-natal days. These authors found a similar increase in skull length and a downward-forward growth of the viscerocranium against the neurocranium (Noda et al., 1994). Fujita et al. performed orchiectiomies on male rats at five postnatal days and studied lateral cephalographs to determine effects on craniofacial growth. This procedure, the removal of the source of endogenous androgen, resulted in inhibition of calvarial growth, especially of the nasomaxillary and mandibular areas (Fujita et al., 2004). This suggests that androgens influence cranial growth after birth, not just during pubertal growth spurt. The association between high levels of androgen, or the absence of androgen and its affect on craniofacial growth indicates that

this systemic factor may play a role in both normal suture development and that deemed pathological.

Androgen receptors are also present in cranial sutures. Lin et al. (2004) demonstrated the presence of androgen receptors in dura mater and in cells of the osteogenic fronts and in the sutural mesenchyme of late gestation mice. These authors suggest that androgens can modulate calvarial growth via binding to receptors in osteoblasts and dural cells. Additionally, they found androgen receptors in osteoblasts in the osteogenic front of human fetal calvaria. This suggests that androgen receptors may promote osteogenesis in these calvarial bones. In addition, any effect androgen may have on the craniofacial skeleton is most likely caused by levels of circulating serum androgens rather than the level of androgen expression (Lin et al., 2004). These same authors also demonstrated that fetal calvarial osteoblasts and dural cells showed increased proliferation and differentiation after androgenic hormone expression suggesting a possible role in suture fusion (Lin et al., 2007). What role excess androgens have in mediating suture fusion in cases where there is a genetic predisposition for craniofacial anomalies necessitates investigation. The absence of research concerning these interactions is due to lack of a congenitally affected animal model.

1.4.4 Craniofacial Anomalies

Some craniosynostoses present with a severe aberration in the steroid hormone pathway. Cytochrome P450 oxidoreductase, POR, is described as a flavoprotein that transfers electrons to P450 enzymes, including those on the steroidogenic and cholesterol synthesis pathway (Huang et al., 2005; Miller, 2005; Miller et al., 2005). A combined deficiency in two of these enzymes P450c17 and P450c21 due to a mutation in the *POR* gene results in what has been described as a severe form

of congenital adrenal hyperplasia, Antley-Bixler Syndrome (Adachi et al., 2004a; Adachi et al., 2004b; Marohnic et al., 2006; Shackleton et al., 2004; Sue Masters and Marohnic, 2006). Fgfr2 mutations can also manifest the skeletal anomalies of Antley-Bixler. Thus, Fgfr2 may be a driving factor in the craniofacial components of the disease. However, recent research has suggested a decoupling of the FGFR2 mutation from the sequence (Reardon et al., 2000; Tsai et al., 2001).

Antley-Bixler Syndrome presents with severe impairment of the cholesterol metabolism and hormonal pathway. An affected female is generally born with ambiguous genitalia indicating excess intrauterine androgen exposure. Conversely, an affected male may exhibit poor masculanization, perhaps due to the biphasic nature of the effects of sex steroids on growth. The disorder has a hallmark accumulation of steroid metabolites, specifically 17-hydroxylase and 21-hydroxylase. This in turn causes a stunting of the steroidogenic pathway leading to a deficiency in cortisol, affecting the adrenal cortex. In addition, excess metabolites of pregnenolone and progesterone accumulate due to the block in the pathway to the sex steroids affecting their target cells (Fluck and Miller, 2006; Fluck et al., 2004; Huang et al., 2005; Kelley et al., 2002; Miller et al., 2005; Porter, 2003; Scott et al., 2007).

Antley-Bixler Syndrome, the most severe of the disorders on the *POR* mutation spectrum, is an autosomal recessive disorder with poor epidemiology for incidence, owing to the rare nature of the disease. Most cases are however sporadic in nature (Adachi et al., 2004a; Adachi et al., 2004b; Al-Hassnan and Teebi, 2007). The presence of severe congenital craniofacial and skeletal anomalies characterizes Antley-Bixler syndrome. The most commonly described are craniosynostosis resulting in trapezoidocephaly (early fusion of coronal and lambdoid sutures), severe midface hypoplasia, choanal stenosis, and synostosis of the radiohumeral joint (Arlt, 2007; Fukami et al., 2005; Machado et al., 2001; Sulaiman et al., 2007). Many medical specialists are required for treatment and maintenance following initial diagnosis including otolaryngologist, medical genetics, neurosurgery, endocrinology and cardiology. Airway management is often the

primary treatment due to choanal atresia, often requiring nasal stints or tracheotomy. Cortisol replacements are generally necessary to prevent further damage to the adrenal cortex. Craniofacial abnormalities require surgical intervention to attempt to correct the synostosed sutures and prevent complication to neural growth (Arlt, 2007; Bradley et al., 2003; Cragun and Hopkin, 2005; Shackleton et al., 2004).

1.5 EXPERIMENTAL ANIMAL MODELING OF CRANIOSYNOSTOSIS

Research has demonstrated that growth of the cranium proceeds perpendicular to a functional suture. Research using animal models has utilized experimental immobilization of the coronal, sagittal and interfrontal sutures for growth studies, and to model surgical corrections. For example, Babler utilized roentgenocephalometrics to determine the growth trajectories after coronal suture immobilization. Although these animals demonstrated normal somatic growth, they suffered significant alterations in craniofacial growth. Inhibition of growth across the affected sutures occurred, in addition, to shortening of the cranial vault length and height. The anterior lambdoid site also exhibited a decreased growth. In contrast, the parietotemporal suture exhibited compensatory growth. Shortening of the cranial base lengths occurred as well. The surgical manipulations, via strip suturectomy suggested that earlier intervention via surgery could allow for better craniofacial growth trajectories (Babler and Persing, 1982; Babler et al., 1982; Persing et al., 1981). Losken et al., also experimentally immobilized the coronel suture to model bilateral craniosynostosis. Craniectomy alone or coupled with frontal bone advancement

allowed for release of the affected suture. Results demonstrated resynostosis and growth disturbances with craniectomy only intervention, with better growth results for the frontal bone advancement, advocating for this surgical intervention, clinically (Losken et al., 1991a; Losken et al., 1991b).

Researchers utilize the posterior interfrontal suture, a normally fusing suture in mouse models to study craniosynostosis. Opperman et al. utilized this natural model to investigate the delivery of TGFβ3 via a collagen vehicle to the posterior interfrontal suture by periosteal tunnel. Results demonstrated a greater frequency of patent sutures in the TGFβ3 treated group. This suggests local TGFβ3 in the extracellular matrix may be necessary for suture patency (Opperman et al., 2002). Utilizing a similar model, Cooper et al., investigated postoperative re-synostosis using the posterior interfrontal suture in mice. These investigators performed suturectomies on the interfrontal sutures of experimental mice and delivered Noggin, a BMP antagonist via a collagen gel to the extirpated suture area. Results suggested Noggin successfully inhibited resynostsosis in these animals, suggesting a possible future direction for these complications in craniosynostosis clinical cases (Cooper et al., 2009).

Genetic mouse models have also become increasingly popular for the study of craniosynostosis. Models readily identified in the literature include an Apert model (Hajihosseini et al., 2001; Holmes et al., 2009; Shukla et al., 2007; Tanimoto et al., 2004; Twigg et al., 2009; Wang et al., 2005; Yang et al., 2008; Yin et al., 2008; Yu and Ornitz, 2001), Crouzon model (Carlton et al., 1998; Chen et al., 2003; Perlyn et al., 2006), Saethre-Chotzen model (Connerney et al., 2006; Firulli et al., 2005), Muenke model (Mansour et al., 2009), and MSX2 related model (Satokata et al., 2000). Problems exist for the identification of these models as correlates to human syndromes as many are specific to the genetic loci and not phenotype, which historically

has been how human syndromes are characterized (Cohen and MacLean, 2000; Cohen, 2005). To date these studies have concentrated on producing the models, and description of the phenotype. Exploration is necessary to determine the clinical usefulness of these models.

A rabbit model with congenital synostosis of the coronal suture has been described (Mooney et al., 1994a; Mooney et al., 1994b; Mooney et al., 1998b; Mooney et al., 1998c). Similar to humans, this colony of New Zealand White Rabbits demonstrates autosomal dominant transmission with incomplete penetrance (Mooney et al., 1996). The model also presents with a broad range of phenotypic expression (from unilaterally affected animals, animals with delayed-onset suture synostosis, to animals presenting with complete bilateral fusion) (Mooney et al., 1998b; Mooney et al., 1998c), **Figure 7**. These affected rabbits seem to be over expressing Msx2 at the suture site (Horutz et al., 1996) as well as TGFβ2 (Poisson et al., 2004), suggesting that the same gene(s) or pathways are involved in this pathogenesis as human syndromes. Successful post-suturectomy interventions for craniofacial growth, intracranial volume, and prevention of resynostosis have utilized Noggin and an anti-TGFβ2 antibody (Cooper et al., 2007; Mooney et al., 2007a; Mooney et al., 2007b). In addition, a successful rescue, delivery of TGFβ3 to the cranial suture via periosteal tunnel in a collagen vehicle, prior to fusion has been described (Chong et al., 2003). The rabbits, because they possess susceptible phenotypes, provide a unique opportunity for investigating the relationship between systemic variations in circulating growth factors, hormones and congenital suture pathology.

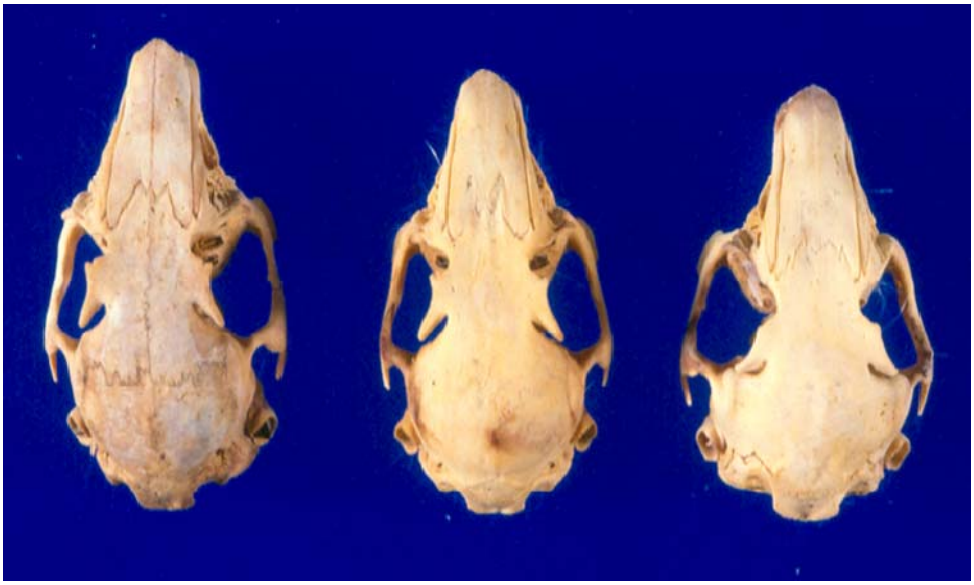

Figure 7: Craniosynostotic Rabbit Model

From left to right: Wild Type, Delayed Onset Synostosis, Early Onset Synostosis

Modified from (Mooney et al., 2007b)

1.6 STATEMENT OF PURPOSE, HYPOTHESIS AND GOALS

The purpose of this dissertation project was to examine how the endocrine system, specifically androgenic hormone, interacts with suture growth and synostosis. The affected craniosynostotic rabbit model (early onset synostosis and delayed onset synostosis) was used to investigate several hypotheses:

1) A cellular level response was investigated using normal rabbit suture and non-suture bone cells as controls and early onset synostotic suture and non-suture cells as experimental groups. These cells were exposed to differing concentrations of testosterone to elicit cell proliferation and differentiation responses. The hypothesis given results obtained by Lin et al.

(Lin et al., 2004; Lin et al., 2007) and the known effect of testosterone on TGFβ, was that an increase in proliferation and differentiation would occur due to testosterone exposure to these suture and non-suture bone cells, in a dose dependent manner. In addition, a co-culture of testosterone and BMP was conducted to better characterize this interaction. It was hypothesized that the co-culture would lead to a decrease in proliferation due to BMPs known effect on the osteoblast lineage, and an even greater increase in differentiation, than testosterone treatment alone, in a dose dependent manner.

2) Flutamide, an anilide, which competes with testosterone and dihydrotestosterone for binding to androgen receptors, thus acting as an androgen blocker (Reynolds, 1996) was also be used to characterize the effects of testosterone and BMP co-cultures on these cells. It was hypothesized that flutamide administration would mediate/block the effects testosterone has on these cells decreasing proliferation and differentiation relative to the expected increases, in a dose dependent manner.

3) Western blotting and immunohistochemistry was utilized to detect the presence of androgen receptors in tissues and cells harvested from normal wild type New Zealand white rabbit, and the affected synostotic rabbits.

4) Finally, an in vivo study utilized an androgen receptor blocker, flutamide, to rescue the suture of delayed onset synostosis affected rabbits, destined to undergo suture synostosis. Currently the etiopathogenesis of non-syndromic craniosynostosis in humans is poorly understood (Cohen and MacLean, 2000; Cohen, 2005). Sex hormones are a possible endogenous variable that can be modified by the environment, or may present as purely environment exposure, thus acting as a teratogen affecting craniofacial development (Herbst, 1973; Limbird and Taylor, 1998; Molsted et al., 1997). A better understanding of the gene-environment

interaction could aid in diagnosis and management of craniofacial anomalies (Murray, 2002). The synostotic rabbits, because they possess susceptible phenotypes, provide a unique opportunity for investigating the relationship between systemic variations in circulating androgen, androgen receptor activity, and congenital suture pathology. If the androgenic pathways are perturbed, clinical or subclinical cases of excess androgen could potentially interfere with TGFβ therapies currently designed to treat craniosynostosis (Chong et al., 2003; Mooney et al., 2007a; Mooney et al., 2007b). Lin et al., (Lin et al., 2004; Lin et al., 2007) reported on the identification of androgen receptors presence in the osteogenic fronts and underlying dura of several cranial sutures in late gestation fetal mice. This provides the basis for the effect of androgens in suture growth and morphology. It was hypothesized that delivery of flutamide to the coronal suture could delay osseous fusion and facilitate greater growth across the coronal suture and correction of craniofacial growth in this craniosynostotic model.

2.0 MATERIAL AND METHODS

2.1 PRIMARY RABBIT BONE CELL TESTOSTERONE CHALLENGE

Tissue was harvested from 10 day old New Zealand White Rabbits, both wild type and those expressing the congenital coronal suture fusion phenotype born into the breeding colony at the University of Pittsburgh, Department of Anthropology. This model has been previously described (Mooney et al., 1994a; Mooney et al., 1994b), and similar to humans, this colony of New Zealand White Rabbits demonstrates autosomal dominant transmission with incomplete penetrance. Tissue was harvested from the coronal sutures of the rabbits as well as the parietal bone for non-suture bone control. Cells from the coronal suture and non suture parietal bone were isolated and grown in T-75 flasks in Dulbecco's modified Eagle's medium (DMEM, supplemented with penicillin/streptomyacin (pen/strep) and fetal bovine serum (FBS). At passage 2 cells were seeded in 96 well plates in triplicate at a density of 1,000 cells per well, based on cell density studies in which the optimal density was determined to prevent cell confluence and allow for adequate growing across 7 days of treatment. Each animal was studied for cell proliferation and differentiation as per methodology described below.

MC3T3-E1 murine osteoprogenitor cells, harvested from murine calvaria and purchased as a stock immortalized cell line (American Type Culture Collection (ATCC), Manassas, VA), were utilized for the purpose of study consistency, error bars indicate of accuracy in cell culture,

and as a control for whether activity should be expected. Cells were reconstituted and grown in a T-75 flask until transfer into T-175 flask. Cells were cultured in an alpha minimum Eagles medium (αMEM) which contains L-Glutamine, Phenol Red and Sodium Pyruvate. The αMEM was supplemented with streptomyacin (pen/strep) and fetal bovine serum (FBS). These cells were seeded in 96 well plates in triplicate at a density of 10,000 cells per well, based on cell density studies in which the optimal density was determined to prevent cell confluence and allow for adequate growing across 7 days of treatment. The MC3T3-E1 served as the positive control for the cell assay studies.

Treatments

Testosterone propionate (TP) was used as an experimental treatment on the cell lines. TP was purchased from Sigma Aldrich (St. Louis, MO). TP has a molecular weight of 344.5. 100% Ethanol was used to reconstitute the TP, and titrations were employed to create the concentrations used for experimental study.

Control and experimental treatment wells were set up for each plate, treatments consisted of: proliferation media, basic control cells seeded and fed basic media; ethanol, control for testosterone reconstitution; Bone Morphogenetic Protein-4 at 5ng/ml, a polypeptide belonging to the TGFβ superfamily which plays a strong role in bone development; TP reconstituted at concentrations of -12, -14,-16,-20,-24,and -30 molecular weight; and TP concentrations added to BMP4 at 5ng/ml. Treatments were run in triplicate wells (averaged) and the studies were run in or in excess of triplicate resulting in N=3 or greater for each treatment per cell type.

Assessment of Proliferation

Cell proliferation was determined by *Cell-titer 96 aqueous one solution cell proliferation assay kit* (Promega, Madison, WI, USA). After 7 days of treatment, cells seeded into the 96-well

plates were incubated for one hour with 20 µl of *Cell-titer 96 aqueous one solution* added to each well. The absorbance at 490 nm was recorded with a 96-well plate reader (Biotek, VT).

Assessment of Differentiation

Cell differentiation was determined by an alkaline phosphatase (ALP) activity assay. ALP is an early biochemical marker for osteoblast differentiation. After 7 days of treatment, media was removed from cells seeded into the 96 well plates and cell lysis was performed. After 30 minutes of incubation at 4°C deionized water and a p-Nitrophenyl phosphate solution was added to the lysis buffer. Three control wells containing no cells were also treated. Plates were then incubated at room temperature in the dark for 30 minutes. The absorbance at 405 nm was recorded with a 96-well plate reader (Biotek, VT). ALP activity was then calculated using the following formula: ((Optical Density – the mean Optical density of the control wells)*total volume*dilution) / (18.45*sample volume). All statistical analyses were performed using SPPS 15.0 (Chicago, IL).

2.2 PRIMARY RABBIT BONE CELL FLUTAMIDE TREATMENT

Tissue was harvested from 10 day old New Zealand White Rabbits, both wild type and those expressing the congenital coronal suture fusion phenotype born into the breeding colony at the University of Pittsburgh, Department of Anthropology. This model has been previously described (Mooney et al. 1994a; 1994b), and similar to humans, this colony of New Zealand White Rabbits demonstrates autosomal dominant transmission with incomplete penetrance. Tissue was harvested from the coronal sutures of the rabbits as well as the parietal bone for non-

suture bone control. Cells from the coronal suture and non suture bone were isolated and grown in T-75 flasks in Dulbecco's modified Eagle's medium (DMEM, supplemented with penicillin/streptomyacin (pen/strep) and fetal bovine serum (FBS). At passage 2 cells were seeded in 96 well plates in triplicate at a density of 1,000 cells per well, based on cell density studies in which the optimal density was determined to prevent cell confluence and allow for adequate growing across 7 days of treatment. Each animal in the study was studied for cell proliferation and differentiation as per methodology described below.

MC3T3-E1 murine osteoprogenitor cells, harvested from murine calvaria and purchased as a stock immortalized cell line (American Type Culture Collection (ATCC), Manassas, VA). Cells were reconstituted and grown in a T-75 flask until transfer into T-175 flask. Cell were cultured in an alpha minimum Eagles medium (αMEM) which contains L-Glutamine, Phenol Red and Sodium Pyruvate. The αMEM was supplemented with /streptomyacin (pen/strep) and fetal bovine serum (FBS). These cells were seeded in 96 well plates in triplicate at a density of 10,000 cells per well, based on cell density studies in which the optimal density was determined to prevent cell confluence and allow for adequate growing across 7 days of treatment. The MC3T3-E1 served as the positive control for the cell assay studies.

Treatments

Testosterone propionate (TP) and flutamide, an androgen receptor blocker, were used as experimental treatment on the cell lines. TP and flutamide were purchased from Sigma Aldrich (St. Louis, MO). TP has a molecular weight of 344.5; flutamide has a molecular weight of 276.2. 100% Ethanol was used to reconstitute the TP and flutamide, and titrations were employed to create the concentrations used for experimental study. Control and experimental treatment wells were set up for each plate, treatments consisted of: proliferation media, basic control cells seeded

and fed basic media; ethanol, control for testosterone reconstitution; Bone Morphogenetic Protein-4 at 5ng/ml, a polypeptide belonging to the TGFβ superfamily which plays a strong role in bone development; TP reconstituted at a concentrations of -16, molecular weight, TP at -16 molecular weight added to BMP4 at 5ng/ml, flutamide at concentrations of -8, -10, and -12 molecular weight, flutamide concentrations added to TP at -16 molecular weight, and flutamide concentrations added to TP at -16 molecular weight, and BMP4 at 5ng/ml. Treatments were run in triplicate wells (averaged) and the studies were run in or in excess of triplicate resulting in N=3 or greater for each treatment per cell type.

Assessment of Proliferation

Cell proliferation was determined by *Cell-titer 96 aqueous one solution cell proliferation assay kit* (Promega, Madison, WI, USA). After 7 days of treatment, cells seeded into the 96-well plates were incubated for one hour with 20 µl of *Cell-titer 96 aqueous one solution* added to each well. The absorbance at 490 nm was recorded with a 96-well plate reader (Biotek, VT).

Assessment of Differentiation

Cell differentiation was determined by an alkaline phosphatase (ALP) activity assay. ALP is an early biochemical marker for osteoblast differentiation. After 7 days of treatment, media was removed from cells seeded into the 96 well plates and cell lysis was performed. After 30 minutes of incubation at 4°C deionized water and a p-Nitrophenyl phosphate solution was added to the lysis buffer. Three control wells containing no cells were also treated. Plates were then incubated at room temperature in the dark for 30 minutes. The absorbance at 405 nm was recorded with a 96-well plate reader (Biotek, VT). ALP activity was then calculated using the following formula: ((Optical Density – the mean Optical density of the control wells)*total

volume*dilution) / (18.45*sample volume). All statistical analyses were performed using SPPS 15.0 (Chicago, IL).

2.3 IMMUNOHISTOCHEMISTRY

Bone tissue from the coronal suture and non suture parietal bone control in a 10 day old wild-type (WT) and a rabbit with delayed onset (DOCS) and an early onset craniosynostosis rabbit (EOCS), and matched gonads and gonadal fat were be harvested as part of an on-going protocol by Dr. Gregory Cooper, Ph.D. and his lab at Children's Hospital of Pittsburgh in the Department of Pediatric Plastic Surgery. The gonads/gonadal fat were treated as positive controls and were stained first to ensure the usefulness of the primary antibody, as the antibody was specific to human androgen receptor mapping (Xq11.2-q12). A primary antibody, a mouse monoclonal antibody specific to human AR (SantaCruz Technology, CA, cat #sc-52309) used to determine expression of AR, was chosen because it was the only identified antibody not raised in rabbits. This antibody was also chosen because it does not cross-react with estrogen, progesterone, or glucocorticoid receptors, and has been proven to react with AR in human reproductive organ tissues. However the reported cross reactivity against rabbit was less than 2%.

Sections (8–10 μm thick) were deparaffinized, rehydrated through graded alcohols, and treated with deionized water for 3 minutes. Endogenous peroxidase activity was quenched using a 20 minute incubation with 3% H_2O_2 in methanol. Slides were blocked with 2% goat serum, the host of the secondary antibody, through a 30 minute incubation at room temperature. The primary antibody mixed in 2% serum, at dilutions of 1:50, 1:250, or 1:1000, was added and

slides were incubated for 30 minutes at room temperature. Negative control sections were incubated with 2% goat serum with no primary antibody. Sections were then washed in deionized water and secondary antibody, goat anti-mouse IgG (SantaCruz Technology, CA) in phosphate-buffered saline (PBS), was added at dilution 1:250 and 1:500 and incubated for 30 minutes. After the secondary antibody, the presence of AR specific antibody was visualized using diaminobenzidine substrate (Sigma-Aldrich Corp., St. Louis, MO), and the slides washed in water. Sections were then counterstained with Harris hematoxylin, dehydrated, and mounted. Patterns of AR expression of the gonad were examined.

2.4 WESTERN BLOTTING

2.4.1 Western Blotting for Androgen Receptor

Bone cells at the coronal suture, and the parietal bone, non suture bone, in 10 day old wild-type (WT) and rabbits with early onset craniosynostosis (EOCS) have been harvested as part of an on-going protocol by Dr. Gregory Cooper, Ph.D. and his lab at Children's Hospital of Pittsburgh in the Department of Pediatric Plastic Surgery. In addition, stock cultures immortalized mouse calvarial pre-osteoblast cell MC3T3-E1 (E1) were used as a cellular control. Cells were plated and cultured for 3 days in medium. Protein extracts will be prepared as follows. Cold lysis buffer (50 mM Tris–HCl, pH 7.4) containing 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% TritonX 100 and a cocktail of protease inhibitors (Sigma, St. Louis, MO) were added and mechanical homogenization will be performed by five passages through 1 mL pipette tip. The solution was cleared by centrifugation (5000 × g for 15 s). Protein concentrations

were assayed using the Bradford method, using known protein concentration to determine a standard curve (Zor and Selinger, 1996). (10-15 µl of Laemmli buffer added to a microtube, add beta mercaptoethanol at 5% of total sample volume, protein sample was added at 20 µg. Sample were heated for 2 minutes at 70 degrees Celsius). Proteins were separated by 10% SDS-PAGE and then electro-blotted onto a nitrocellulose membrane. The membrane was soaked for 1 h in PBST (PBS, 0.05% Tween 20) containing 5% BSA. Rabbit cells were then incubated overnight at 4 °C with primary monoclonal mouse human specific AR antibody (SantaCruz Technology, CA, cat # sc-52309,) (1:250), primary polyclonal AR antibody raised in rabbit (Santa Cruz Technology, CA, cat # sc-815) (1:250) or beta actin control primary antibody raised in mouse (ABCAM Technology, MA, cat # ab6276) (1:1000). MC3T3-E1 were incubated overnight with the same primary antibodies. Membranes were then washed three times (15 min) with PBS, incubated with secondary peroxidase-labeled IgG (1:10,000) specific to primary antibody (Mouse primary antibodies subjected to Goat anti-mouse secondary; rabbit primary antibody subjected to Goat anti-rabbit secondary antibody) (LICOR Odyssey Systems, NE) for 1 h and washed three times with PBS. Bound antibodies were detected by using the ultraviolet western blotting detection kit according to the manufacturer's recommendations, Odyssey Infrared Imaging System. Molecular weights of proteins were determined using biotinylated protein markers.

2.4.2 Coomassie Blue Total Protein Stain

A coomassie blue stain was performed to identify total protein at specific molecular weights at both stages of Western blotting protocol prior to incubation with antibody, after SDS protein gel electrophoresis and blotting membrane transfer (see above). Due to the questionable

usefulness of the primary antibody as described above, this step was used as a non-specific indicator of presence of protein about the level where the androgen receptor would be identified at 137 kd (R & D systems, Santa Cruz Biotechnology). The coomassie blue treatment allows the visualization of protein bands. Following the above western protocol 20 μg of protein from wild type and congenital synostosed non suture bone and suture bone as well as MC3T3-E1 cells were subjected to SDS Page electrophoresis and blotting membrane transfer. Resulting gel and membrane were stained with coomasie blue stain for 15 minutes while shaking. Destaining was performed with a solution 40% methanol, 10% acetic acid, 50% deionized water. Destaining solution was left on for 45 minutes while shaking, and destaining solution was switched out for fresh solution every 15 minutes. A protein molecular weight marker, ladder, was used to determine the region of interest (BIORAD Kaleidoscope, CA). Images were captures via digital camera, gel reader, and desktop scanning for best resolution.

2.5 IN-VIVO FLUTAMIDE TREATMENT FOR DELAYED ONSET CRANIOSYNOSTOTIC USING A NON-SYNDROMIC RABBIT MODEL

Sample sizes of at least six per group are based on power calculations from a previous study, with the calculated effect size of 0.80, and setting alpha at 0.05. New Zealand White rabbits (Oryctolagus cuniculus), born in our ongoing breeding colony of congenitally synostosed animals (Mooney et al., 1994a; Mooney et al., 1994b) **Figure 8**, were utilized in the study. Rabbits were randomly assigned to four groups as follows: Group 1- rabbits with delayed onset synostosis and periosteal elevation (surgical control group), n=10; Group 2- rabbits with delayed onset synostosis, periosteal elevation, and collagen and bovine serum albumin (BSA) protein

(vehicle control group 1), n=6; Group 3- rabbits with delayed onset synostosis, periosteal elevation, and collagen and 100% ethanol (vehicle control group 2), n=6; Group 4- rabbits with delayed onset synostosis, periosteal elevation, and collagen with 15mg of flutamide dissolved in 100% ETOH (Experimental Group), n=8.

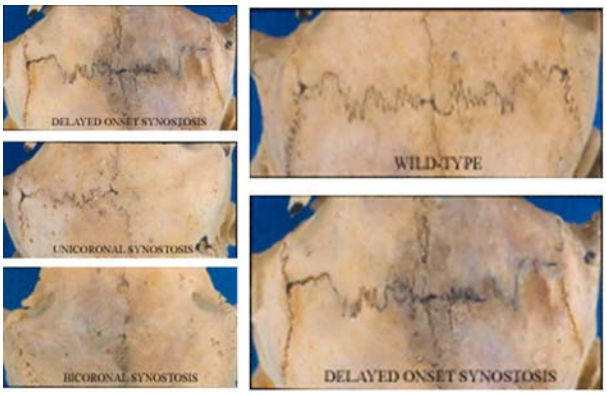

Figure 8: Craniosynostotic Rabbit Sutures

Provided by Dr. Mark Mooney

At 10 days of age, rabbits were anesthetized with an IM injection (0.59 ml/kg) of a solution of 91% Ketaset (Ketamine Hydrochloride, 100 mg/ml) and 9% Rompun (Xylazine, 20 mg/ml). The scalps will then be shaved, depilated, scrubbed with betadine/alcohol**,** and prepared for sterile surgery. The calvaria were exposed using a midline scalp incision and the skin reflected laterally to the supraorbital borders. Holes were then be made in the periosteum and bone using a fine dental bur (0.4 mm) and packed with silver dental amalgam to serve as radiopaque markers. The holes were placed in quadrants, 3mm lateral to the sagittal suture, 3mm anterior and posterior to the coronal, frontonasal, and anterior lambdoidal sutures, **Figure 9**. At 25 days of age the initial diagnosis was reassessed and confirmed from pre-operative and 25 day radiographs and the rabbits with delayed onset craniosynostosis were randomly assigned to the aforementioned groups. The rabbits were anesthetized with an IM injection (0.59 ml/kg) of a

solution of 91% Ketaset (Ketamine Hydrochloride, 100 mg/ml) and 9% Rompun (Xylazine, 20 mg/ml), the scalps were prepared for sterile surgery, and calvaria exposed for surgery. In rabbits in Group 1, periosteal tunnels overlying the coronal suture were created, and nothing was injected, **Figure 10a**. In rabbits in Group 2, 0.1ml of collagen, **Figure 10b**, containing 500ng of BSA was injected into the periosteal tunnels overlying the coronal suture. In rabbits in Group 3, .1 ml of collagen was mixed with 100% ETOH and was injected into the periosteal tunnels overlying the coronal suture. In rabbits in Group 4, .1 ml of collagen was mixed with 15 mg flutamide (Sigma Aldrich, St. Louis), dissolved in 100% ETOH. The scalp wounds were then closed with 4/0 Vicryl (resorbable) sutures. All animals received postoperative SQ injections (2 mg/kg) of Baytril (Bayer Corp., Shawnee Mission, KS 66201) BID for 5 days as a prophylaxis for infection. Following surgery, pups were taken off the table and returned to the prep/recovery room.

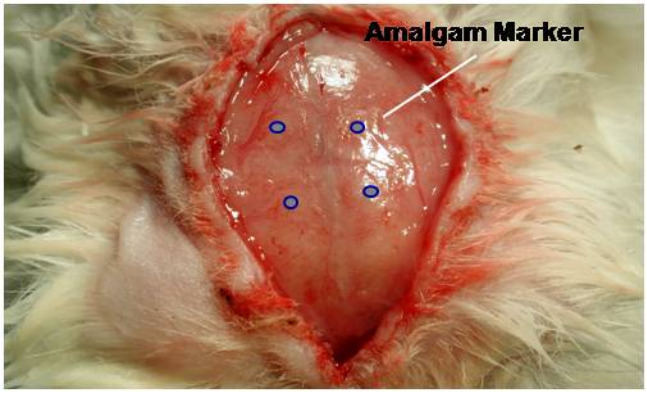

Figure 9: Figure 9: Amalgam Markers Implanted in the Skull

Provided by Dr. Mark Mooney

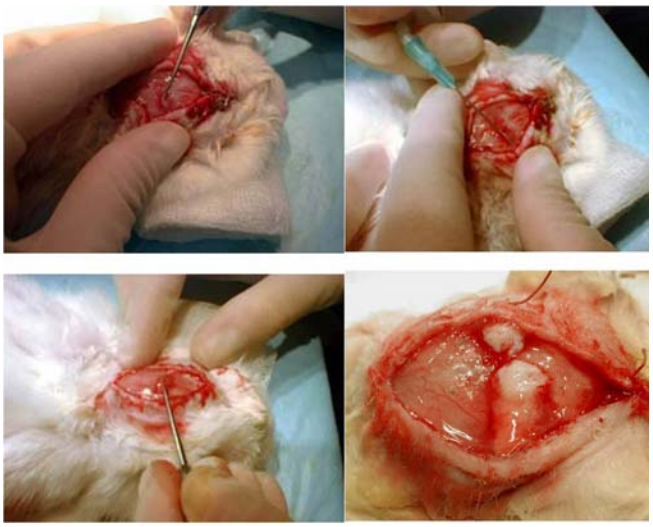

Figure 10: Periosteal Tunnel Overlying the Coronal Suture

10a on left: Periosteal Tunnels; 10b on right: Collagen Vehicle

Provided by Dr. Mark Mooney

Cranial vault growth was assessed and monitored at 10, 25, 42, and 84 days of age using serial head radiographs. The coronal sutures from each group of rabbits were harvested at 84 days for any future histomorphometry or immunohistochemical studies. Means and standard deviations for the various craniofacial growth variables were calculated and compared among conditions using a 3 x 4 (group by age), two-way analysis of variance, with a repeated measure design. Intergroup differences will be assessed using least squared differences comparison test. Mean differences will be considered significant if $p<0.05$.

Serial lateral and dorsoventral head radiographs (including the front right paw) were taken with the rabbits sedated with an intramuscular injection (0.40 mL/kg) of a solution of 91% Ketaset (ketamine hydrochloride, 100 mg/mL, Aveco Co., Inc.) and 9% Rompun (xylazine, 20 mg/mL; Mobay Corp.). The heads were immobilized in a specifically designed cephalostat, **Figure 11**, and a Phillips Oralix 70 (Washington, DC) dental x-ray unit was used at an exposure

of 50 kV, 7 mA and a .17- to .50-second exposure time, and a tube-to-cassette distance was held constant at 152 cm. The cephalographs were viewed on a light box and a number of cephalometric landmarks and amalgam markers on either side of the coronal suturectomy site were identified and traced on acetate tracing paper. The tracings were then scanned using a Microtek 9800 XL scanner and the digital images were stored on a Dell Optiplex PC. The landmarks were assigned Cartesian (x and y) coordinates and the distances between the markers were measured using the Dolphin image analysis software program (Dolphin Imaging & Management Solutions, Sacramento, CA). The landmarks and markers that were identified from the cephalographs included: 1) CS, anterior and posterior coronal suture markers; 2) ALS, anterior lambdoidal suture; 3)MOP, maximum occipital point; 4) OP, opisthion; 5) SOS, sphenooccipital synchondrosis; 6) FE, frontoethmoidal point; 7) UMP, upper molar point; 8) PR, prosthion; 9) PSES, presphenoethmoidal synchondrosis; and 10) the proximal and distal articular surfaces of the third, right metacarpals viewed on the dorsoventral cephalographs. Somatic, sutural, and craniofacial growth were assessed by calculating age-related changes in a number of measures, including bodyweight and third metacarpal length; amalgam marker separation at the coronal suturectomy site; overall craniofacial length (MOP-PR); intracranial volume (cranial length*cranial width*cranial height); cranial base length (SOS-PSES); and the cranial vault shape (height/length) index ([ALSSOS/OP-FE]*100); cranial length (OP-FE); cranial width (widest vault point); cranial height (ALS-SOS); cranial base angle (BAS-OF-FE °); and palatal angle (BAS-OF-PR °), **Figure 12**. All of the measurements were performed blind with regard to group identity. A random sample of 10% of past radiographs were traced and measured twice. Intraobserver, repeated measurement reliability was calculated at $r = 0.963$ ($p < 0.01$) with a

3.15% standard error of measurement. All statistical analyses were performed using SPPS 15.0 (Chicago, IL).

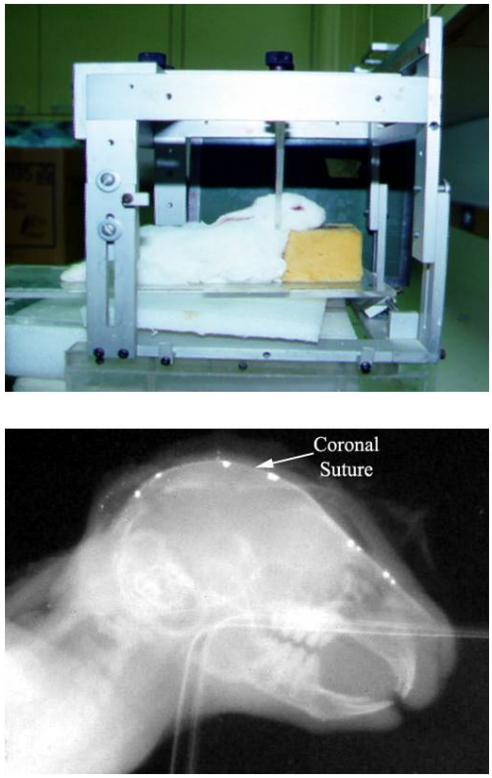

Figure 11: Cephalostat and 25 day Radiograph with Amalgam Markers

Provided by Dr. Mark Mooney

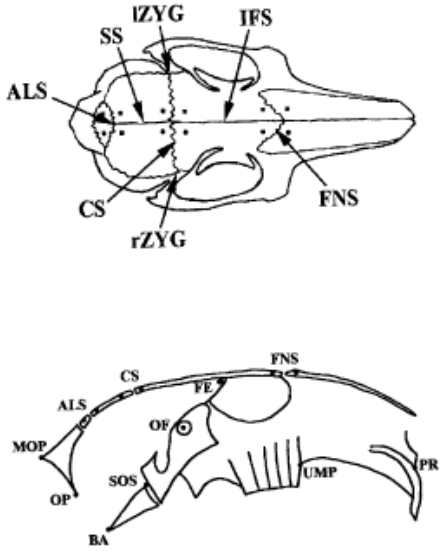

Figure 12: Cephalometric Landmarks

Provided by Dr. Mark Mooney

3.0 RESULTS

3.1 CELLULAR TESTOSTERONE CHALLENGE

3.1.1 Assessment of Proliferation

Table 1 provides the sample sizes for these analyses. The MC3T3-E1 are included in the graphs for cell control representation, especially to denote if differences in activity are to be expected. Note the small error bars. It appears as though the MC3T3-E1 lack a proliferative response to BMP alone, but do react to testosterone treatments. However, there appears to be a lack of response for the BMP+Testosterone co-cultures.

Table 1. Sample Sizes for Proliferation of Testosterone Study

Cell Type	N
Congenital Synostosed Suture Bone	7
Congenital Synostosed Non Suture Bone	3
Wild Type Suture Bone	4
Wild Type Non Suture Bone	3
MC3T3-E1	3

To determine the effects of reconstituting the stock hormones in ethanol, a pair design was employed to determine the relationship between the proliferation response between cells treated with proliferation media only and proliferation media with ethanol added. The assumption of normality was met for each cell type for both treatments. There was a significant difference between proliferation media response (χ=.5419, SD .2124) and proliferation media with ethanol added (χ=.5047, SD .1993), t=2.704, df=16, p=.016. Thus comparison of hormone only response will be normalized to proliferation media with ethanol added.

To determine of adding BMP resulted in altered proliferation activity a paired design was employed comparing proliferation media only and proliferation media with BMP added. The assumption of normality was met for both PM and BMP. There was no significant difference in proliferative response between proliferation media (χ=.5419, SD .2124) and proliferation media with BMP added (χ=.5545, SD .2042), t= .448, df=16, p= .660.

The proportion of BMP proliferative activity, normalized to proliferation media response, was investigated for differences by cell type. Means are represented in **Figure 13**. Synostotic cells were the most responsive with non suture bone having the greatest mean response. These cells were also the most variable. A two way ANOVA design was implemented for BMP proliferative response by phenotype (wild versus synostotic) and cell type (suture versus non suture bone). The assumption of normality was violated for synostotic non-suture bone affected cell types (non-suture bone: W= 0.764, p= .031). A natural log transformation allowed for normality across cell types. The assumption of homogeneity of variance was violated, F= 4.87, df 3,13, p=.018. ANOVA should be robust against this violation, however, susceptibility to type I errors are noted. There was no significant difference for BMP proliferative response by phenotype by cell type, F=0.880, df. 3, 13, p=.365. There was no significant difference in BMP

proliferative response by phenotype averaged across cell type, F= 2.411, df 1, 13, p=.144. There was no significant difference in BMP proliferative response by cell type averaged across phenotype, F= 0.006, df 1, 13, p=.940.

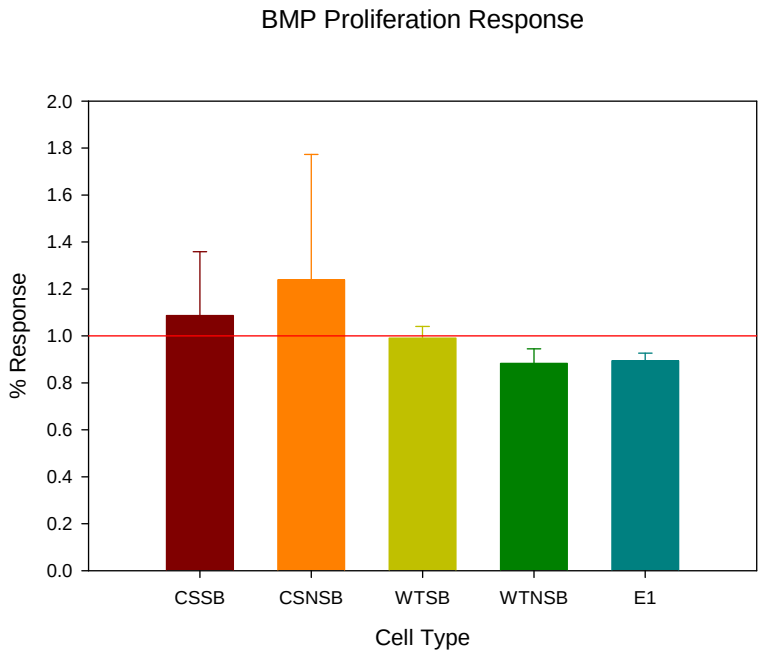

Figure 13: BMP Proliferative Response by Cell Type

(1.0 indicates 100% of baseline proliferation media response)

To determine if adding testosterone resulted in altered proliferation activity a paired design was employed comparing proliferation media with ethanol added and proliferation with ethanol and testosterone added. The assumption of normality was violated for both groups. After transformations, the natural log performed best, however both groups still violated normality (ETOH: W=.848, p<.001; Testosterone: W=.910, p<.001). Thus a one sample Wilcoxon test was applied. There was no significant difference in proliferative response between proliferation media with ethanol added (χ=.5047, SD .1943) and proliferation media with ethanol and testosterone added (χ=.5274, SD .2020), Z= -1.838, p=.066.

The proliferative response of cells treated with testosterone was investigated for dose (-12, -14,-16,-20,-24, and -30 mols.) response, phenotype and cell type by a three way analysis of variance. Violations of normality occurred for synostotic phenotype suture bone cells at -12,-20, -24, and -30 dosages. An inverse transformation resulted in the best fit for normality, but violations occurred for the same categories, W=.793, df=7, p=.040, for each respectively. The dose response of the testosterone treatments by cell type normalized to activity of proliferation media with ethanol added are illustrated in **Figures 14-15**. It appears that the synostotic cell types demonstrate the greatest relative proliferative response, especially at lower dosages. The wild type suture bone mean response does not reach 100% of proliferation with ethanol added activity except at the lowest dose, thus non suture bone cell types appear to have more proliferative reaction. Note the large standard errors indicating inherent variability. The assumption of homogeneity of variance was met F=1.487, df 23, 78, p=.101. The pattern of differences for proliferative response by dose by phenotype by cell type, F=.005, df 5, 78, p=1.000. The pattern of difference of cell type by dose averaged across phenotype was no significant, F=.106, df 5, 78, p=.991. The pattern of difference of phenotype by dose averaged across cell type was not significant F=.055, df 5, 78, p=.998. The pattern of difference of phenotype by cell type averaged across dose was not significant, F=1.084, df 1, 78, p=.301. The pattern of differences of dose averaged across phenotype and cell type, was not significantly different, F=.191, df 5, 78, p=.965. The pattern of difference by cell type averaged across phenotype and dose was not significantly different, F=.019, df 1, 78, p=.892. The pattern of difference by phenotype averaged across dose and cell type was not significantly different F=3.236, df 1, 78, p=.076.

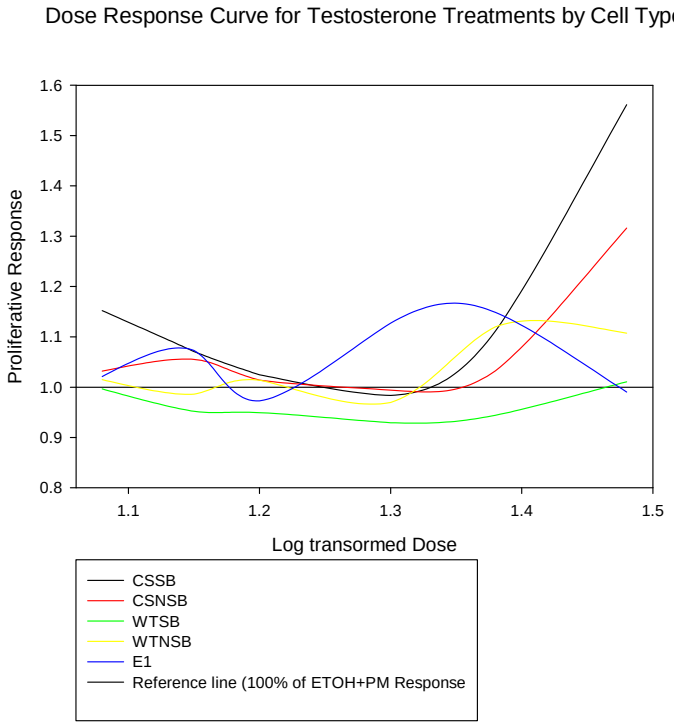

Figure 14: Dose Response Curve for Proliferation Activity with Testosterone Added Normalized to Proliferation Media+ETOH Proliferation Activity.

Reference line indicates 100% of Baseline Proliferation Activity of Proliferation Media with Ethanol Added

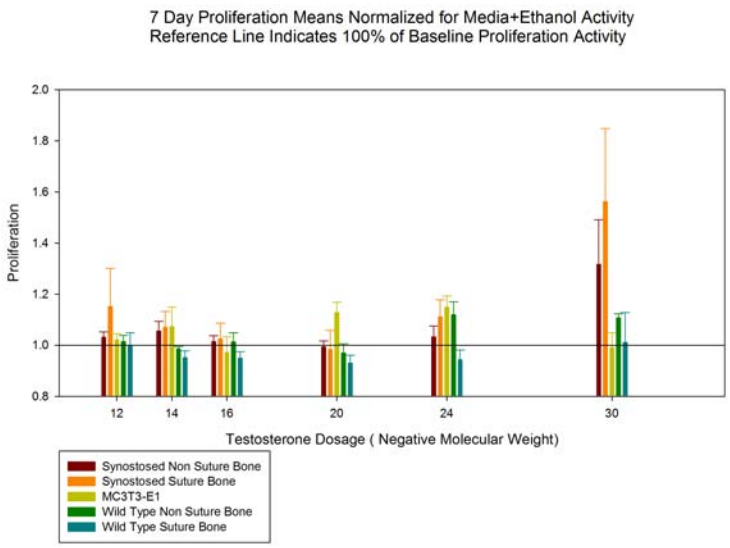

Figure 15: Proliferation Response Means. Normalized for Proliferation Activity Treated with Media Only

Reference line indicates 100% of Baseline Proliferation Activity of Proliferation Media with Ethanol Added

To determine if adding testosterone to BMP resulted in altered proliferative activity a paired design was employed comparing proliferation media with BMP added and proliferation media with testosterone and BMP added. The assumption of normality was violated for proliferation media with BMP added data ($W=.917$, $p<.001$). Transformations did not fare better. Thus a one sample Wilcoxon test was applied. There was no significant difference in proliferation response between proliferation media with BMP added ($\chi=.5545$, $SD=.1991$) and proliferation media with BMP and testosterone added ($\chi=.6067$, $SD=.1960$), $Z= -.249$, $p= .804$.

The proliferative response of cells treated with testosterone added to BMP 50 ng/mol normalized to baseline BMP proliferative activity was investigated for dose (-12, -14, -16, -20, -24, and -30 mols.) response, phenotype and cell type, by a three way analysis of variance. **Figures 16-17** exhibit the dose responses for proliferation with testosterone treatment co-cultured with BMP. The synostotic cell types appear to have a much greater proliferative

response, with wild type means not reaching baseline BMP proliferative activity levels. The synostotic cells appear to have a biphasic response with highest doses and lowest doses exhibiting greater activity than intermediate doses. Note the large standard errors indicating inherent variability. The assumption of normality was violated for all doses of synostotic suture bone responses and wild type suture bone at -14 doses. An inverse transformation allowed for normality for all categories with exception of wild type suture bone at -14 mols, W=.749, p=.04. The assumption of homogeneity of variance was violated F=2.238, df 23, 74, p=.005. The susceptibility to committing a type 1 error is noted due to this violation. The pattern of difference for testosterone+BMP proliferative response by dose by phenotype by bone type were not significantly different, F= .787, df 5, 78, p= .562. The pattern of difference of phenotype by dose averaged across bone type were not significantly different, F= 0.977, df 5, 78, p=.437. The pattern of difference of bone type by dose averaged across phenotype was not significantly different, F= 0.496, df 5, 78, p= .778. The pattern of difference of phenotype by bone type averaged across dose was not significantly different, F=0.545, df 1, 78, p=.463. There were no statistically significant differences for proliferation by dose averaged across phenotype and bone type, F= 1.372, df 5, 78 p=.244. There were no significant differences by cell type averaged across phenotype and dose, F= 0.579, df 1, 78, p= .449. There was a significant difference in proliferative response by phenotype averaged across cell type and dose, F=12.171, df 1, 78, p=.001. Synostotic phenotype (χ=1.446, SD 0.834) exhibited much greater overall proliferation normalized to baseline BMP proliferation compared to wild type phenotype (χ= 0.924, SD 0.082).

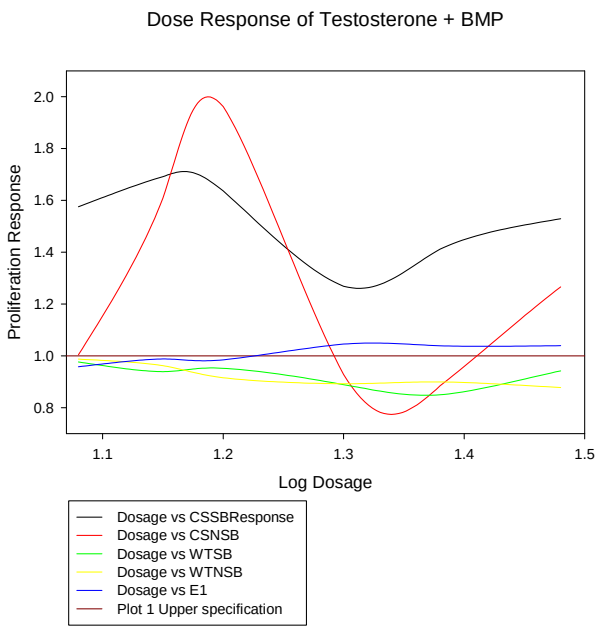

Figure 16: Dose Response Curve for Proliferation Activity with Testosterone Added to BMP Normalized to Proliferation Media+BMP Proliferation Activity

Reference line indicates 100% of Baseline Proliferation Activity of Proliferation Media with BMP Added

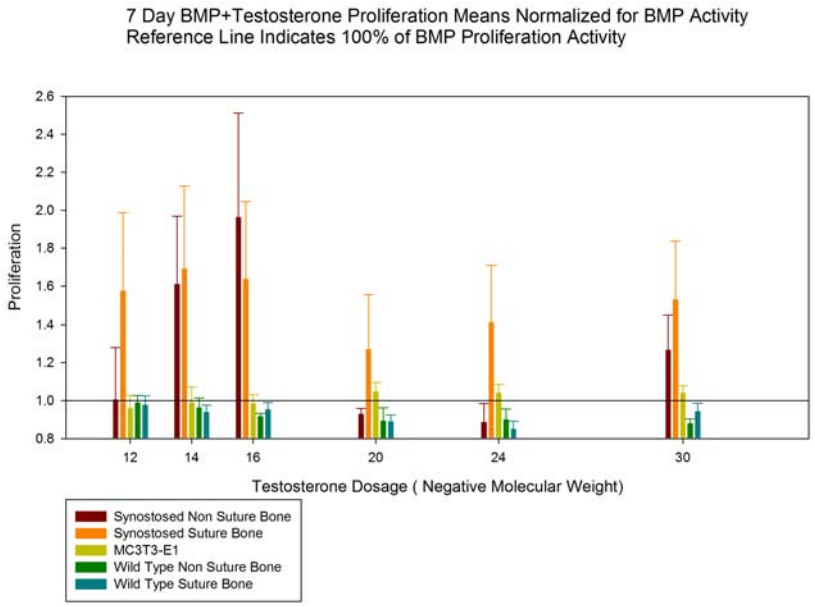

Figure 17: Proliferation Response Means. Normalized for Proliferation Activity Treated with Proliferation Media with BMP Added Means

Reference line indicates 100% of Baseline Proliferation Activity of Proliferation Media with BMP Added

3.1.2 Assessment of Differentiation

Table 2 provides the sample sizes for these analyses. The MC3T3-E1 are included in graphs for cell control representation, especially to denote if activity should be expected. Note the small errors bars. It appears as though the MC3T3-E1 are very responsive BMP alone, but much less so compared to other cell types for testosterone and BMP and testosterone co-cultures. **Figure 18** are the representative ALP stains indicating differentiation, for each cell type for baseline proliferation media treatment, BMP treatment, testosterone treatment and testosterone with BMP added treatment. Note all are positive, indicating the necessity for the quantitative ALP analyses below.

Table 2: Sample Sizes for Differentiation of Testosterone Study

Cell Type	N
Congenital Synostosed Suture Bone	7
Congenital Synostosed Non Suture Bone	3
Wild Type Suture Bone	4
Wild Type Non Suture Bone	3
MC3T3-E1	3

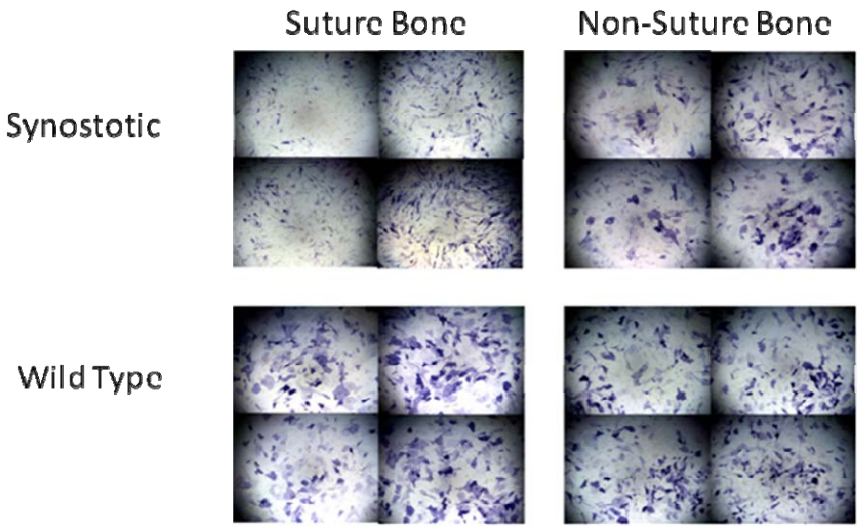

Figure 18: Alkaline Phosphate Stain for Cell Types

For each composite starting on the top left moving clockwise: Proliferation Media Treatment, BMP Treatment, Testosterone Treatment, Testosterone BMP Co-culture Treatment

To determine the effects of reconstituting the stock hormone in ethanol, a pair design was employed to determine the relationship between the differentiation response between cells treated with proliferation media only and proliferation media with ethanol added. The assumption of normality was violated for both proliferation media only and proliferation media with ethanol added for differentiation response (PM: W=0.796, p=.001; PM+ETOH=0.806, p=.001). Square root, inverse, natural log, log based 10 and squared transformation failed to normalize the data. Thus a one sample Wilcoxon test was applied. There was a significant difference between proliferation media response (χ=0.0415, SD .0486) and proliferation media with ethanol added (χ=0.0322, SD=0.0407), Z= -2.059, p=.039, with PM+ETOH exhibiting less differentiation response. The comparison of hormone only response will be normalized to proliferation media with ethanol added.

To determine if adding BMP resulted in altered differentiation activity a paired design was employed comparing proliferation media only and proliferation media with BMP added. The assumption normality was violated for both PM and BMP. After transformations, the natural log performed best normalizing the BMP data. However proliferation media still violated normality (PM: W=.855, p=.013). Thus a one sample Wilcoxon test was applied. There was no significant difference in differentiation response for between proliferation media (χ=0.0415, SD .0486) and proliferation media with BMP added (χ=0.0559, SD=.0649), Z= -1.823, p=.068.

The proportion of BMP differentiation activity, normalized to proliferation media response, was investigated for differences by cell type. Means are represented in **Figure 19**. Synostotic cells had the greatest variability, but the greatest response, especially non suture bone compared to the wild type phenotype. A two way ANOVA design was implemented for BMP differentiation by phenotype (wild versus synostotic) and cell type (suture vs. non suture bone). The assumption of normality was violated for both synostotic cell types, suture (W= 0.768, p=.020) and non suture bone (W=0.735, p=.028). A natural log transformation allowed for greater normality with only the non suture bone cell type violating normality (W= 0.725, p=0.22). The assumption of homogeneity of variance was met, F=1.568, df 3, 13, p=.245. Figure 6 demonstrates the means for each group. Note the large standard errors indicating inherent variability. The histogram suggests wild type suture bone to have the greatest response, with both synostotic cell types being fairly consistent and showing less activity, and wild type non suture bone showing the least response. There was no significant pattern of differences for BMP response for phenotype by cell type, F= 2.151, df 1, 13, p= .166. There were no significant differences in BMP response by phenotype averaged across cell type, F=.144, df 1, 13, p=.711.

There were no significant differences in BMP response by cell type averaged across phenotype, F= 0.035, df 1, 13, p=.854.

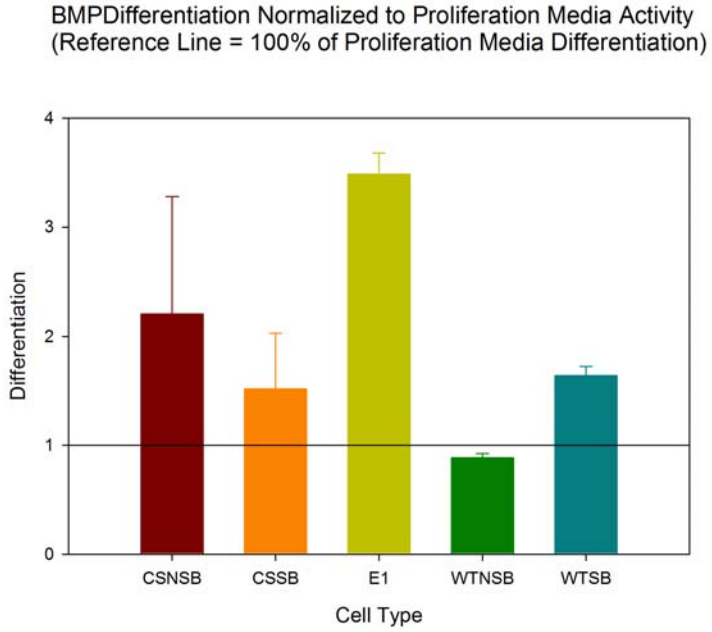

Figure 19: BMP Differentiation Activity Normalized to Proliferation Media Response

(1.0 indicates 100% of baseline proliferation media response)

To determine if adding testosterone resulted in altered differentiation activity a paired design was employed comparing proliferation media with ethanol added and proliferation media with ethanol and testosterone added. The assumption normality was violated for both groups. After transformations, the natural log performed best, however both groups still violated normality (ETOH: W=.933, p<.001; Testosterone: W=.913, p<.001). Thus a one sample Wilcoxon test was applied. There was no significant difference in differentiation response for between proliferation media with ethanol added (χ=0.0322, SD .0397) and proliferation media with ethanol and testosterone added (χ=0.0344, SD=.0356), Z= -0.976, p=.326.

The differentiation response of cell treated with testosterone was investigated for dose (12, -14, -16, -20, -24, and -30 mols.) response, phenotype and cell type by a three way analysis

of variance. Violations of normality occur for the following groups, synostotic suture bone at -12 dose, -14 dose, -24 and -30 dose, synostotic-non suture bone at -30 doses, and wild type suture bone at -24 and -30 dosages. A natural log transformation resulted in the best fit for normality (square root, inverse, log base 10, squared) with violations occurring for synostotic suture bone at -24 dose (W=.801, p=.042) and -30 dose (W=.703, p=.004) and synostotic non suture bone at -30 dose (W=.666, p=.004). Dose response curve, **Figures 20-22**, demonstrated the greatest reaction from the synostotic non suture bone cells with a biphasic response, differentiation peaking at -14 and -30 dosages, with over twenty times the baseline response at its peak. The synostotic suture bone also appears to have a greater reaction than other cell types with a similar biphasic reaction, with close to five times the baseline response at its peak. Note the large standard errors indicating inherent variability. The assumption of homogeneity of variance was violated F=2.185, df 23, 78, p=.013. ANOVA should be robust against this violation. The pattern of difference for differentiation response by dose by phenotype by bone type were not significantly different, F= .425, df 5, 78, p= .830. The pattern of difference of phenotype by dose averaged across bone type were not significantly different, F= .297, df 5, 78, p=.913. The pattern of difference of bone type by dose averaged across phenotype was not significantly different, F= .838, df 5, 78, p= .527. The pattern of difference of phenotype by bone type averaged across dose was significantly different, F=4.916, df 1, 78, p=.030. Here the wild type phenotype exhibited greater differentiation for suture bone (χ=1.228, SD 1.505) than non suture bone (χ= .8820, SD .3079) compared to synostotic phenotype which exhibited much greater mean differentiation for non suture bone (χ=9.874, SD=16.611) than suture bone (χ=2.871, SD 6.060). There were no statistically significant differences for differentiation by dose averaged across phenotype and bone type, F=1.079, df 5, 78, p=.378. There were no significant differences by bone type

averaged across phenotype and dose, F= 1.872, df 1, 78, p= .175. There was a significant difference in differentiation by phenotype averaged across bone type and dose, F=12.557, df 1, 78, p=.001. The synostotic phenotype (χ=4.972, SD 10.749) exhibited much greater overall differentiation than the wild type phenotype (χ=1.080, SD 1.158).

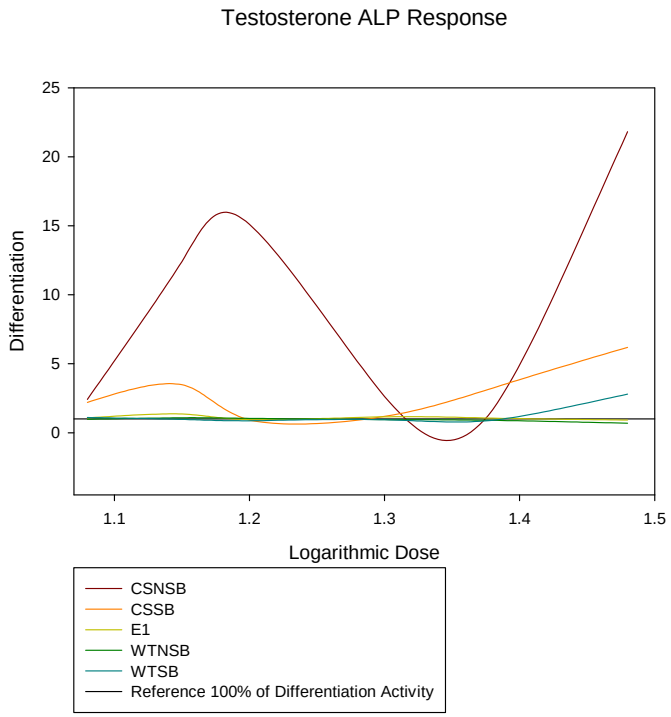

Figure 20: Dose Response for Differentiation Activity with Testosterone Added Normalized to Proliferation Media+ETOH Differentiation Activity

(1.0 indicates 100% of baseline proliferation media with ethanol added response)

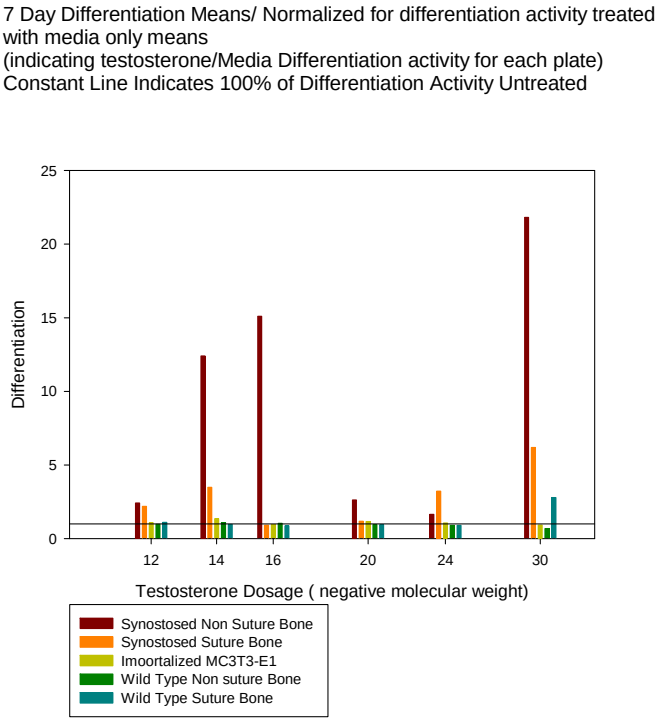

Figure 21: Differentiation Response Means. Normalized for Differentiation Activity Treated with Media Only

Reference line indicates 100% of Baseline Differentiation Activity of Proliferation Media with Ethanol Added

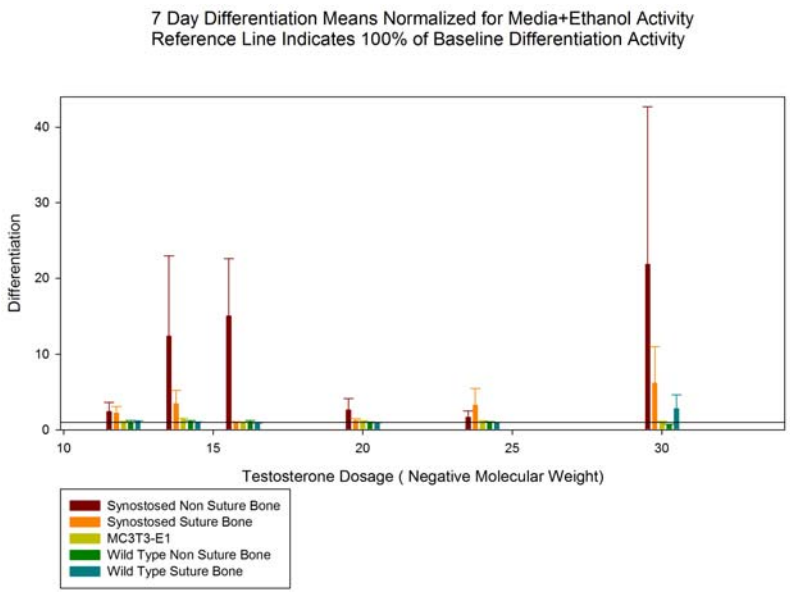

Figure 22: Differentiation Response Means with Error Bars. Normalized for Differentiation Activity Treated with Media Only

Reference line indicates 100% of Baseline Differentiation Activity of Proliferation Media with Ethanol Added

To determine if adding testosterone to BMP resulted in altered differentiation activity a paired design was employed comparing proliferation media with BMP added and proliferation media with testosterone and BMP added. The assumption normality was violated for both groups. After transformations, the natural log performed best, however both groups still violated normality (BMP: W=.891, p<.001; BMP+Testosterone: W=.964, p=.007). Thus a one sample Wilcoxon test was applied. There was a significant difference in differentiation response between proliferation media with bmp added (χ=0.0559, SD .0633) and proliferation media with BMP and testosterone added (χ=0.0577, SD=.0655), Z= -2.493, p=.013, testosterone appearing to increase differentiation than just treatment with BMP alone.

The differentiation response of cell treated with testosterone added to BMP 50 ng/mol, normalized to baseline BMP differentiation activity was investigated for dose (12, -14, -16, -20, -

24, and -30 mols.) response, phenotype and cell type by a three way analysis of variance. **Figures 23-25** exhibit the dose responses for differentiation with testosterone treatments co-cultured with BMP. Synostotic phenotype appears to exhibit a much greater response for differentiation, especially the non suture bone type, peaking at over 40 times baseline activity at the -14 mol treatment and decreasing with decreased dose concentration. Synostotic suture bone also appears to have greater differentiation activity than the remaining cell types, peaking at the -16 mol treatment. Note the large standard errors indicating inherent variability. The assumption of normality was violated for all synostotic permutation and wild type suture bone at -12 and -14 mol treatments. An inverse transformation allowed for normality for all permutation but wild type non suture bone treated with -16 mols, W=.767, p=.039. The assumption of homogeneity of variance was violated F=12.266, df 23, 74, p=.001. The susceptibility to committing a type 1 error is noted due to this violation. The pattern of difference for testosterone+BMP differentiation response by dose by phenotype by bone type were not significantly different, F= .763, df 5, 77, p= .579. The pattern of difference of phenotype by dose averaged across bone type were not significantly different, F= 1.236, df 5, 77, p=.300. The pattern of difference of bone type by dose averaged across phenotype was not significantly different, F= .977, df 5, 77, p= .437. The pattern of difference of phenotype by bone type averaged across dose was not significantly different, F=2.331, df 1, 77, p=.130. There were no statistically significant differences for differentiation by dose averaged across phenotype and bone type, F= .485, df 5, 77 p=.787. There were no significant differences by phenotype averaged across bone type and dose, F= 1.068, df 1, 77, p= .305. There was a significant difference in differentiation by bone type averaged across phenotype and dose, F=5.611, df 1, 77, p=.020. Non suture bone (χ=10.868, SD 29.208) exhibited much greater overall differentiation than suture bone (χ= 3.745, SD 8.444).

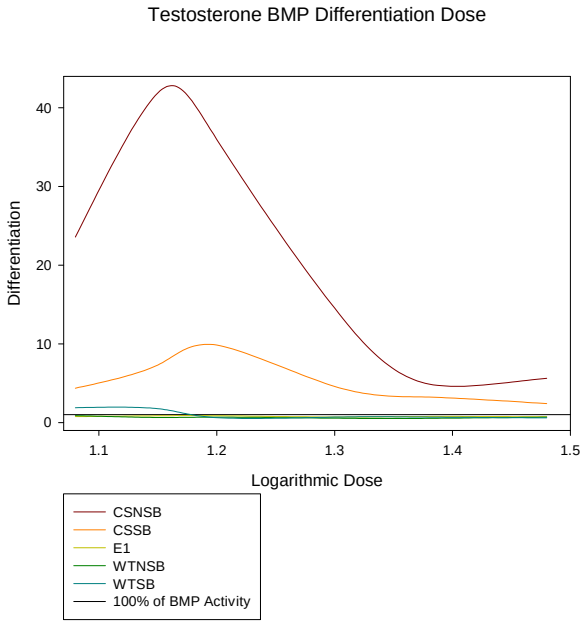

Figure 23: Dose Response for Differentiation Activity with Testosterone BMP Co-Culture Added Normalized to BMP Differentiation Activity

Reference line indicated 100% of Baseline BMP Differentiation Activity

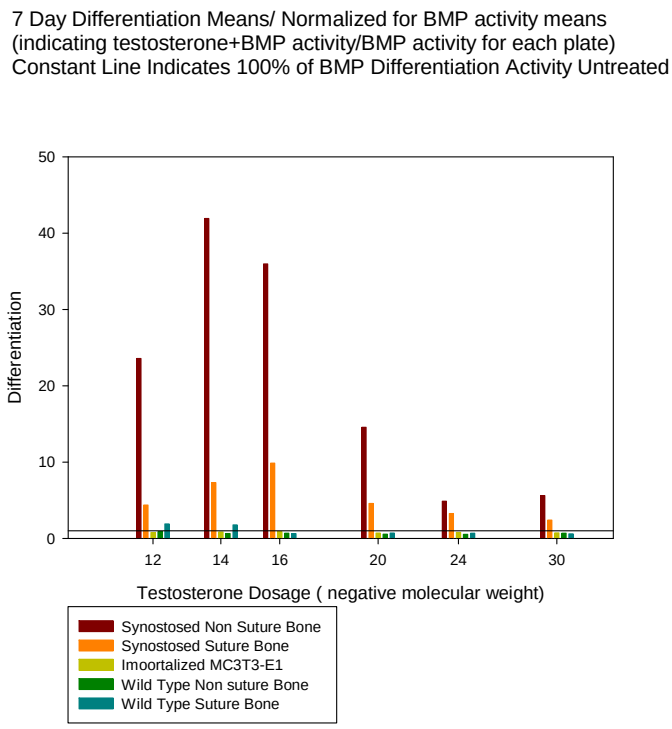

Figure 24: Differentiation Response Means for Differentiation Activity with Testosterone BMP Co-Culture Added Normalized to BMP Differentiation Activity

Reference line indicated 100% of Baseline BMP Differentiation Activity

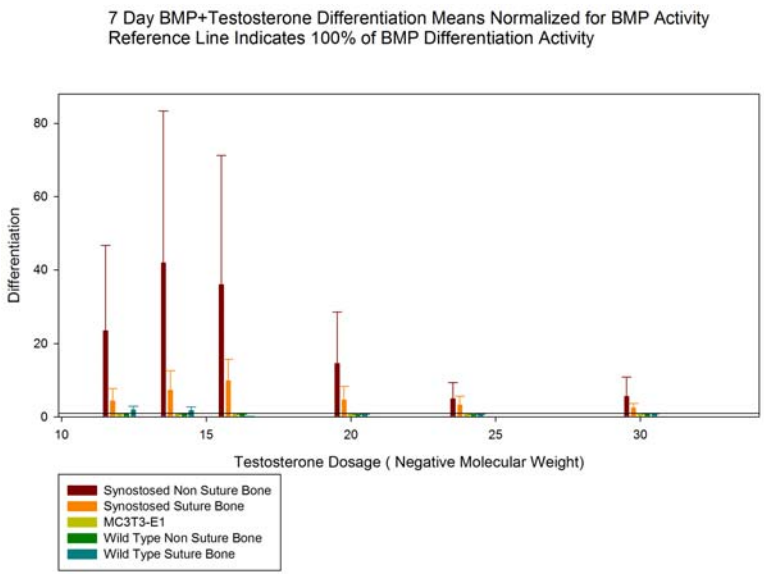

Figure 25: Differentiation Response Means with Error Bars for Differentiation Activity with Testosterone BMP Co-Culture Added Normalized to BMP Differentiation Activity

Reference line indicated 100% of Baseline BMP Differentiation Activity

3.2 CELLULAR FLUTAMIDE TREATMENT

3.2.1 Assessment of Proliferation

Table 3 provides the sample sizes for these analyses. The MC3T3-E1 are included in graphs for cell control representation, especially to denote if activity should be expected. Note the small errors bars. It appears as though the MC3T3-E1 do have responses to the treatments.

Table 3: Sample Sizes for Proliferation of Flutamide Study

Cell Type	N
Congenital Synostosed Suture Bone	6
Congenital Synostosed Non Suture Bone	5
Wild Type Suture Bone	7
Wild Type Non Suture Bone	6
MC3T3-E1	6

To determine the effects of reconstituting the stock hormones in ethanol, a pair design was employed to determine the relationship between the proliferation response between cells treated with proliferation media only and proliferation media with ethanol added. Normality was violated for each treatment. A natural log transformation allowed for normality. There was a significant difference in proliferative response between proliferation media treatment only (χ=.8617, SD .3673) and proliferation media with ethanol added (χ=.8258, SD .3630), t=2.996, df=23, p=.006. The comparison of hormone only response will be normalized to proliferation media with ethanol added.

To determine of adding BMP resulted in altered proliferation activity a paired design was employed comparing proliferation media only and proliferation media with BMP added. The assumption of normality was violated for both groups. A natural log transformation allowed for normality. There was a significant difference in proliferative response between proliferation media treatment only (χ=.8617, SD .3673) and proliferation media with BMP added (χ=.8197, SD .3453), t=2.705, df=23, p=.013. BMP appears to inhibit proliferation activity at 7 day analysis.

The proportion of BMP proliferative activity normalized to proliferation media response was investigated for differences by cell type. **Figure 26** exhibits the means (+/- SE) for each cell type. Wild type cell types appear to exhibit the greatest mean proliferation and the synostotic non suture bone appear to exhibit the least. A two way ANOVA design was implemented for BMP proliferation by phenotype (wild versus synostotic) and cell type (suture versus non suture bone). The assumption of normality was met for all cell types. The assumption of homogeneity of variance was met, F=.404, df 3, 20, p=.752. There was no significant difference for BMP proliferative response for phenotype by cell type, F=.046, df 1, 20, p=.833. There was no significant difference for cell type averaged across phenotype, F=.011, DF 1, 20, p=.918. There was no significant difference for phenotype averaged across cell type, F=1.425, df 1, 20, p=.247.

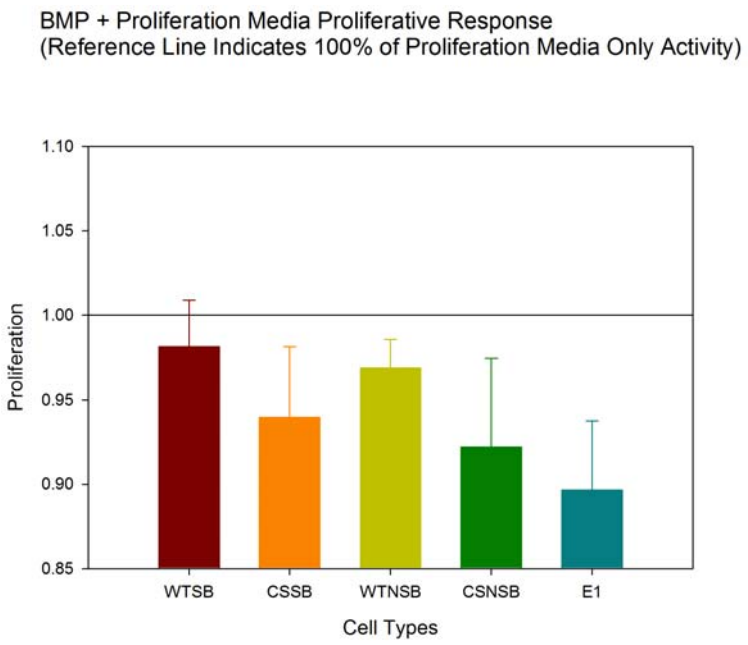

Figure 26: BMP Proliferative Response by Cell Type

(1.0 indicates 100% of baseline proliferation media response)

To determine of adding testosterone 16 mols, resulted in altered proliferative activity a paired design was employed comparing proliferation media with ethanol added and proliferation media with ethanol and testosterone -16 mols added. The assumption of normality was violated for both groups. A natural log transformation allowed for normality for each group. There was a significant difference in proliferative activity between proliferation media with ethanol added (χ=.8258, SD .3630) and Proliferation media with ethanol and testosterone -16 mol treatment added (χ=.7921, SD .3531), t=4.509, df 23, p<.001. Testosterone appears to significantly inhibit the proliferative activity.

The proportion of testosterone -16 mol proliferative activity, normalized to proliferation media with ethanol added, was investigated for differences by cell type. **Figure 27** exhibits the means (+/- SE) for each cell type. All appear to have similar mean response, with some variation about the mean. A two way ANOVA was implemented for T -16 mol proliferation by phenotype (wild versus synostotic) and cell type (suture versus non suture bone). The assumption of normality was met for each type. The assumption of homogeneity of variance was met, F=.204, df 3, 20, p=.893. There was no significant difference for testosterone proliferative response for phenotype by cell type, F=.001, df 1, 20, p=.975. There was no significant difference for cell type averaged across phenotype, F=.081, df 1, 20, p=.778. There was no significant difference for phenotype averaged across cell type, F=0.014, df 1, 20, p=.907.

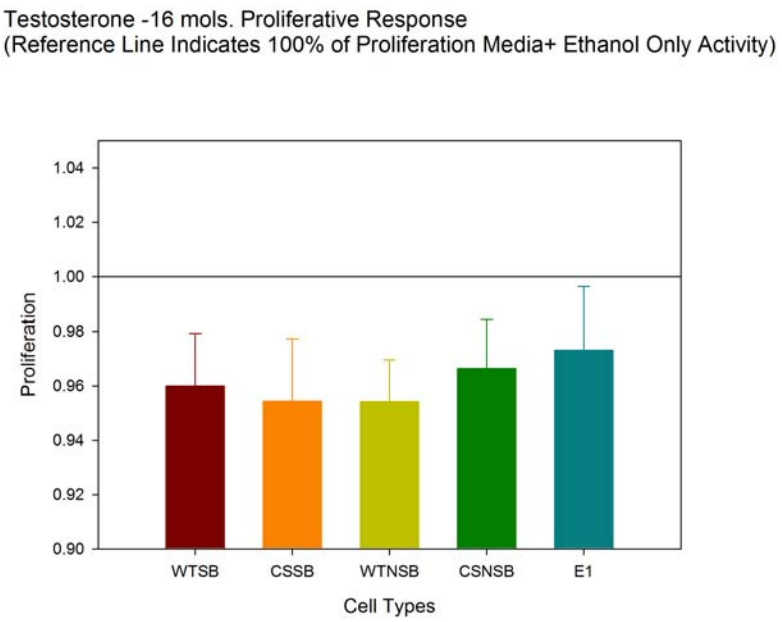

Figure 27: Testosterone -16 mols. Proliferation Activity Normalized to Proliferation Media with Ethanol Response

(1.0 indicates 100% of baseline proliferation media with ethanol added response)

To determine if adding testosterone -16 mols to BMP resulted in altered proliferation activity a paired design was employed comparing proliferation media with BMP added and proliferation with BMP and testosterone added. The assumption of normality was violated for both groups. A natural log transformation allowed for normality for both groups. There was a significant difference in proliferative response with proliferation media with BMP added resulted in greater proliferative activity (χ=.8197, SD .3453) than testosterone added (χ=.7327, SD .2919), t=2.568, df=23, p=.017.

The proliferative response of cells treated with testosterone added to BMP normalized to baseline BMP proliferative response was investigated for difference by phenotype (Wild versus synostotic) and cell type (suture versus non suture bone) by a two way analysis of variance. **Figure 28** represents the mean (+/- SE). Suture bone appears to have a greater proliferative

response than non suture bone types. The assumption of normality was met for all types. The assumption of homogeneity of variance was met, F=.184, df 3, 20, p=.906. There was no significant difference for testosterone +BMP proliferative response for phenotype by cell type, F=.124, df 1, 20, p=.728. There was no significant difference for cell type averaged across phenotype, F=.074, df 1, 20, p=.789. There was no significant difference for phenotype averaged across cell type, F=0.231, df 1, 20, p=.636.

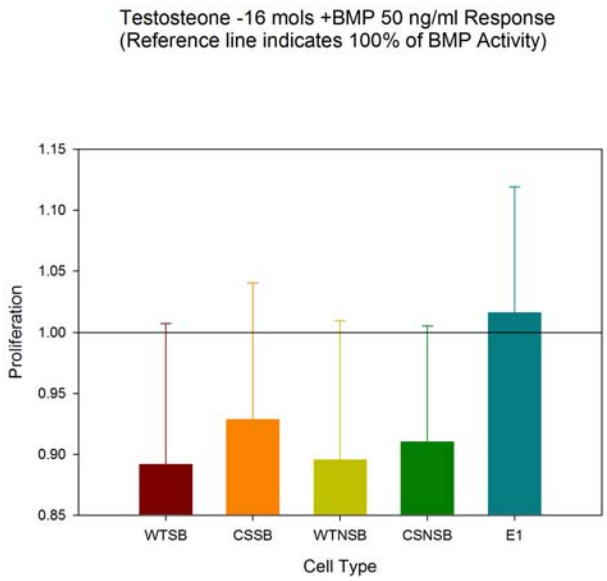

Figure 28: Testosterone -16 mols BMP Co-Culture. Proliferation Activity Normalized to BMP Response

(1.0 indicates 100% of baseline BMP Response)

To determine if adding flutamide resulted in altered proliferation activity a paired design was employed comparing proliferation media with ethanol added and proliferation media with ethanol and flutamide added. The assumption of normality was violated for both treatments. An inverse transformation allowed for normality for each treatment. The proliferation media with

ethanol added showed a significantly higher proliferative response (χ=.8258, SD .3579) than flutamide added (χ=.7533, SD=.3389), t=4.867, df 11, p<.001.

The proliferation response of cells treated with flutamide, normalized to baseline proliferation media with ethanol treatment was investigated for dose (-6, -8, and -10 mols), phenotype (wild type versus synostotic) and cell type (suture versus non suture bone), by a three way analysis of variance. **Figure 29** exhibits the means (+/- SE) for each cell type. With the exception of wild type non suture bone cell treatments, flutamide treatments do not appear to vary greatly in proliferative response. In addition, bone cell types appear to have similar responses. The assumption of normality was met for each type. The assumption of homogeneity of variance was met, F=2.043, df 11, 36, p=.055. There were no significant difference in proliferative response of flutamide by dose by phenotype by cell type, F=.803, df 2, 36, p=.456. There were no significant difference in proliferative response of dose by cell type averaged across phenotypes, F=.181, df 2, 36, p=.835. There was no significant difference by phenotype by dose averaged across cell types, F=1.179, df 2, 36, p=.319. There was no significant difference by phenotype by cell type averaged across doses, F=1.345, df 1, 36, p= .254. There were no significant difference by dose averaged across phenotype and cell type, F=.273, df 2, 36, p=.763. There was no significant difference by cell type averaged across dose and phenotype, F=.000, df 1, 36, p=.986. There was no significant by phenotype averaged across dose and cell type, F=.051, df 1, 36, p=.822.

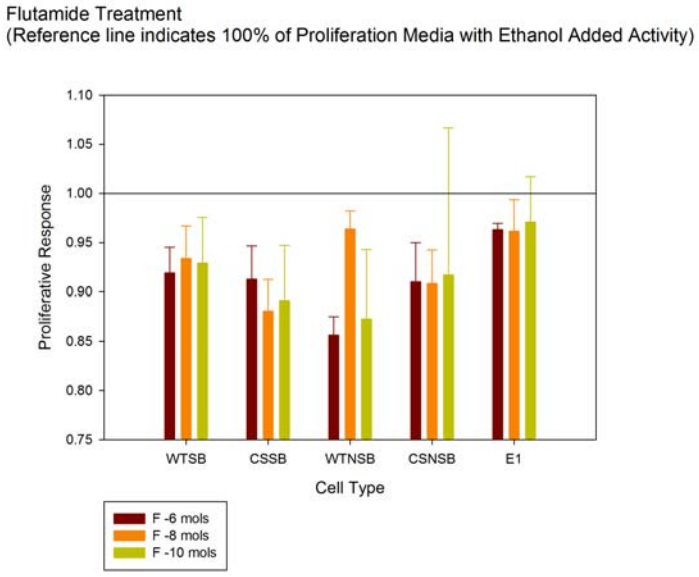

Figure 29: Flutamide Proliferation Activity Normalized to Proliferation Media+Ethanol Response

(1.0 indicates 100% of baseline proliferation media with ethanol added response)

To determine if adding flutamide to BMP resulted in altered proliferation activity a paired design was employed comparing proliferation media with ethanol and flutamide added to BMP, with proliferation media with BMP added only. The assumption of normality was violated for both groups. An inverse transformation allowed for normality across the groups. The BMP added to proliferation media (χ=.8197, SD .3404) exhibited significantly greater proliferative activity than the flutamide + BMP co-culture (χ=.7589, SD .3074), t= 3.516, df 47, p=.001.

The proliferative response of cells treated with flutamide+BMP co-culture, normalized to baseline BMP activity, was investigated for dose response (-6, -8, -10 mols), phenotype (wild versus synostotic) and cell type (suture versus non suture bone), by three way ANOVA. **Figure 30** exhibits the means (+/- SE) for each cell type. There appears to be a dose response across the groups, with the highest concentration of flutamide co-culture reflecting the highest mean proliferative activity. The assumption of normality was violated for the synostotic suture bone at

-8 mols flutamide treatment, W=.743 p=.011. An inverse transformation allowed for normality across groups. The assumption of homogeneity of variance was met, F=1.851, df 11, 36, p=.081. The pattern of differences by dose by phenotype by cell type was not significant F=.157, df 2, 36, p=.855. The pattern of differences of phenotype by dose averaged across cell type were not significant F=1.334, df 2, 36, p=.276. The pattern of differences of phenotype by cell type averaged across dose were not significant, F=.043, df 1, 36, p=.837. The pattern of differences of cell type by dose averaged across phenotype were significant F=3.398, df 2, 36, p=.044. The synostotic cells appears to exhibit a much greater proliferative activity (χ=.7650, SD .2631) at the -10 mols dose than the non suture bone (χ=.5385, SD=.0919). The pattern of differences by cell type averaged across dose and phenotype were not significant, F=1.297, df 1, 36, p=.262. The pattern of differences of phenotype averaged across dose and cell type were not significant, F=.211, df 1, 36, p=.649. The pattern of differences of dose averaged across phenotype and cell type were significant F=5.933, df 2, 36, p=.006. A post hoc pairwise bonferroni comparison revealed the -10 mol treatment (χ=.6706, SD .2333) to exhibit significantly less activity than the -6 mol (χ=.8254, SD .3930; p=.009) and -8 mol treatments (χ=.6706, SD .2944, p=.018).

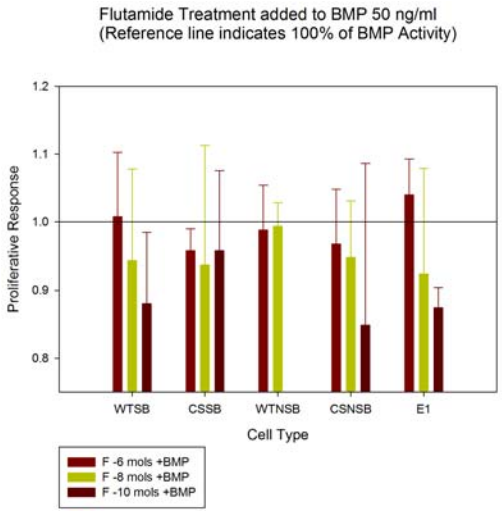

Figure 30: Flutamide + BMP Proliferation Activity Normalized to BMP only response

(1.0 indicates 100% of baseline BMP Response)

To determine if there is an additive in reducing proliferative activity with the addition of flutamide to testosterone in ethanol a paired design was employed comparing the amount of reduction of activity for testosterone and testosterone flutamide co-culture. The assumption of normality was met for both groups. There was no significant difference in the percentage of activity reduced by testosterone (χ=.0417, SD .0452) and flutamide testosterone co-culture (χ=.0386, SD .1327), t=.145, df 47, p=.885.

The percentage of proliferative activity reduced by flutamide co-cultured with testosterone compared to testosterone only treatments were investigated for dose (-6,-8,-10), phenotype (wild type versus synostotic), and cell type (suture versus non suture bone), by a three way ANOVA. **Figure 31** exhibits the means (+/- SE) for each cell type. The assumption of homogeneity of variance was violated for wild type non suture bone at -6 mol dose, W=.759, p=.020. Transformations did not improve on this normality. ANOVA should be robust against this violation. The assumption of homogeneity of variance was met F=2.467, df 11, 36, p=.120.

There were no significant differences by phenotype by cell type by dose, F=1.429, df 2, 36, p=.253. There were no significant pattern of differences of cell type by dose averaged across phenotype, F=.338, df 2, 36, p=.716. There were no significant pattern of differences of phenotype by dose averaged across cell type, F=1.883, df 2, 36, p=.167. There were no significant pattern of differences of phenotype by cell type averaged across dose, F=2.051, df 1, 36, p=.161. There was no significant main effect for dose, F=.465, df 2, 36, p=.632, cell type, F=.055, df 1, 36, p=.816, or phenotype F=.216, df 1, 36, p=.645.

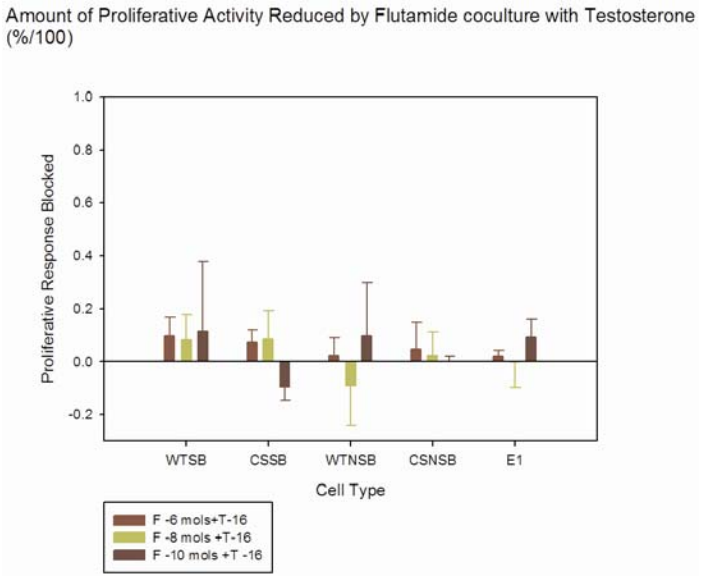

Figure 31: Amount of Proliferative Activity Reduced Flutamide + Testosterone -16 mols.

Proliferation Activity Normalized to Testosterone -16 mols. only response

To determine if there is an additive in reducing proliferative activity with the addition of flutamide to testosterone +BMP a paired design was employed comparing the amount of reduction of activity for testosterone+BMP and testosterone flutamide +BMP co-culture. The assumption of normality was met for both groups. There was a significant difference in the percentage of activity reduced by testosterone added to BMP treatment compared to BMP

baseline (χ=.0943, SD .1050) and flutamide testosterone co-culture with BMP (χ=.1389, SD .1361), t=3.802, df 47, p<.001, suggesting less activity when flutamide is added.

The percentage of proliferative activity reduced by flutamide co-cultured with testosterone and BMP compared to testosterone and BMP only treatments were investigated for dose (-6,-8,-10), phenotype (wild type versus synostotic), and cell type (suture versus non suture bone), by a three way ANOVA. **Figure 32** exhibits the mean response reduced for each cell type (+/- SE). Wild type non suture bone appears to be the most responsive to flutamide treatment. The assumption of normality was violated for wild type non suture bone at -6 mol dose, W=.754, p=.008. Transformations did not improve on this normality. ANOVA should be robust against this violation. The assumption of homogeneity of variance was met F=2.160, df 11, 36, p=.20. There were no significant differences by phenotype by cell type by dose, F=.144, df 2, 36, p=.867. There were no significant pattern of differences of cell type by dose averaged across phenotype, F=1.617, df 2, 36, p=.213. There were no significant pattern of differences of phenotype by dose averaged across cell type, F=1.236, df 2, 36, p=.303. There were no significant pattern of differences of phenotype by cell type averaged across dose, F=1.639, df 1, 36, p=.209. There was no significant main effect for dose, F=1.957, df 2, 36, p=.156, or phenotype F=.302, df 1, 36, p=.586. There was a significant main effect by cell type flutamide blocking less percent activity in suture bone cells (χ=.0235, SD .06883) than non suture bone cells (χ=.0877, SD .1136), F=7.569, df 1, 36, p=.009.

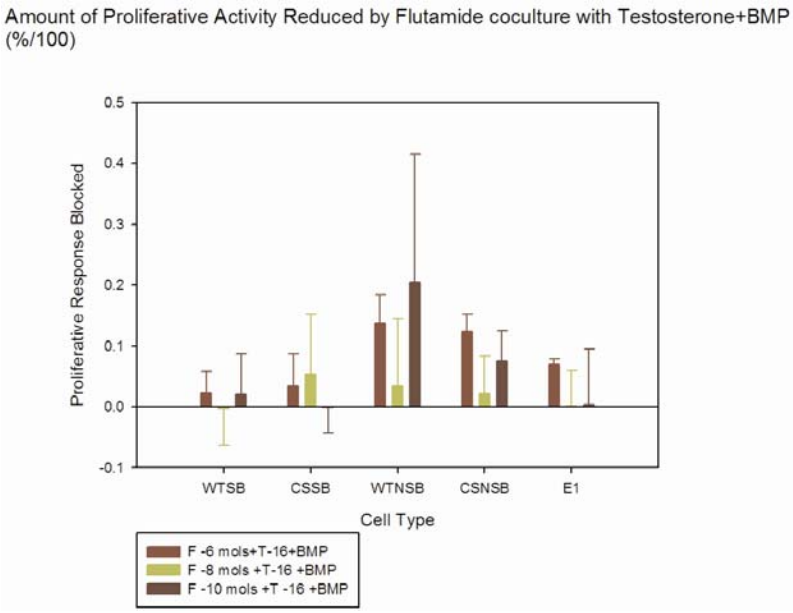

Figure 32: Amount of Proliferative Activity Reduced Flutamide + Testosterone -16 mols BMP Co-Culture

Proliferation Activity Normalized to Testosterone -16 mols. BMP Co-Culture Response

3.2.2 Assessment of Differentiation

Table 4 provides the sample sizes for these analyses. The MC3T3-E1 are included in graphs for control cell representation, especially to denote if activity should be expected. It appears as though these cells are reactive to BMP increasing differentiation, and flutamide decreases differentiation. There does not appear to be a strong response to testosterone treatment. **Figure 33** are the representative ALP stains indicating differentiation, for each cell type for baseline testosterone treatment, testosterone BMP co-culture treatment, flutamide testosterone treatment and flutamide testosterone with BMP added treatment. Note all are positive, indicating the necessity for the quantitative ALP analyses below.

Table 4: Sample Sizes for Differentiation of Flutamide Study

Cell Type	N
Congenital Synostosed Suture Bone	6
Congenital Synostosed Non Suture Bone	5
Wild Type Suture Bone	7
Wild Type Non Suture Bone	6
MC3T3-E1	6

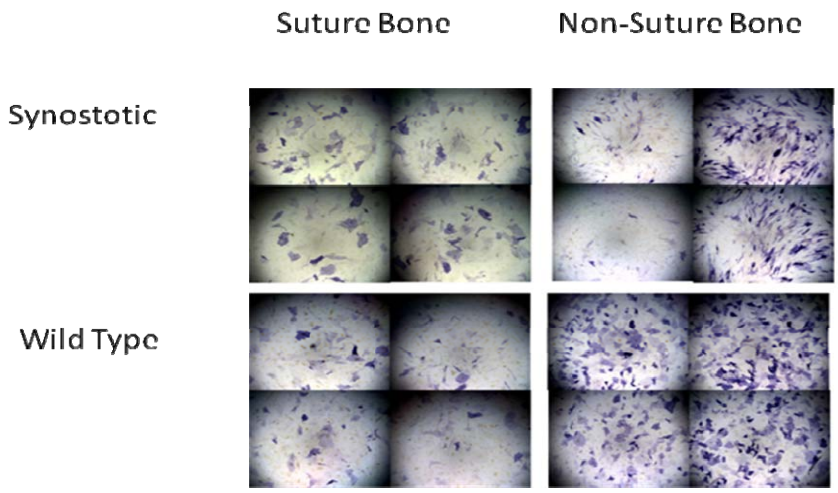

Figure 33: Alkaline Phosphate Stain for Cell Types

For each composite starting on the top left moving clockwise: Testosterone Treatment, Testosterone BMP Co-culture Treatment, Flutamide Testosterone Treatment, Flutamide Testosterone BMP Co-culture Treatment

To determine the effects of reconstituting the stock hormones in ethanol a paired design was employed to determine the relationship between differentiation response between cells treated with proliferation media only and proliferation media with ethanol added. The assumption of normality was violated for both groups. A natural log transformation allowed for normality of both groups. There was a significant difference in differentiation between

proliferation media only (χ=.0273, SD .0168) and proliferation media with ethanol added (χ=.0242, SD .01338), t=3.293, df 23, p=.003. The comparison of hormone only response will be normalized to proliferation media with ethanol added.

To determine if adding BMP resulted in altered differentiation a paired design was employed comparing proliferation media only and proliferation media with BMP added. The assumption of normality was violated for both groups. A natural log transformation allowed for normality for both groups. BMP exhibited greater differentiation (χ=.0434, SD .0408) compared with baseline proliferation media response (χ=.0273, SD .0168), t=3.260, df 23, p=.003.

The proportion of BMP differentiation activity normalized to proliferation media response was investigated for difference by cell type. **Figure 34** exhibits the means (+/- SE) for each cell type. Wild type suture bone appears to have a very great response compared to other cell types. A two way ANOVA design was implemented for BMP differentiation by phenotype (wild versus synostotic) and cell type (suture versus non suture bone). The assumption of normality was violated for wild type suture bone category. A natural log transformation allowed for normality across groups. The assumption of homogeneity of variance was met, F=2.065, df 3, 20, p=.137. There was no significant pattern of differences of phenotype by cell type for BMP differentiation response, F=1.810, df 1, 20, p=.194. There was no significant difference by cell type averaged across phenotype for BMP differentiation, F=2.900, df 1, 20, p=.104. There was no significant difference by phenotype averaged across cell type for BMP differentiation, F= 1.306, df 1, 20, p=.267.

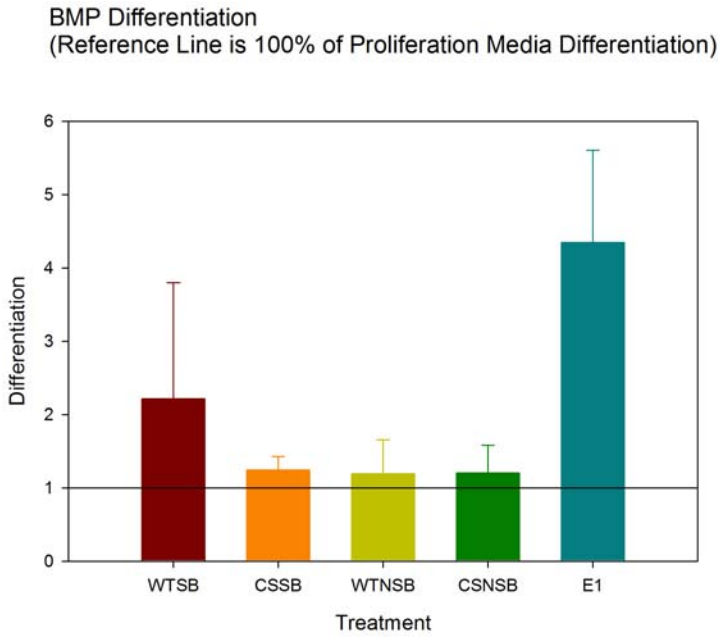

Figure 34: BMP Differentiation Response by Cell Type

(1.0 indicates 100% of baseline proliferation media response)

To determine if adding testosterone -16 mols, resulted in altered differentiation a paired design was employed comparing proliferation media with ethanol added and proliferation with ethanol and testosterone -16 mols added. The assumption of normality was violated for both groups. A natural log transformation allowed for normality for each group. There was no significant difference in differentiation between proliferation media with ethanol added (χ=.0242 SD .0134 and that with T -16 mols added (χ=.0244, SD .0135).

The proportion of testosterone -16 mol differentiation, normalized to proliferation media with ethanol added, was investigated for differences by phenotype (Wild versus Synostotic) and cell type (suture versus non suture bone), by two way ANOVA. **Figure 35** exhibits the means (+/- SE) for each type. Wild type non suture bone appears to have the only mean increase in differentiation, with other types appearing to be about 100% of baseline activity. The assumption of normality was met for all groups. The assumption of homogeneity of variance was met,

F=.673, df 3, 20, p=.579. There was no significant pattern of differences of phenotype by cell type for differentiation, F=.925, df 1, 20, p=.348. There was no significant difference by cell type averaged across phenotype for differentiation, F=2.650, df 1, 20, p=.119. There was no significant difference by phenotype averaged across cell type for differentiation, F=2.092, df 1, 20, p=.164.

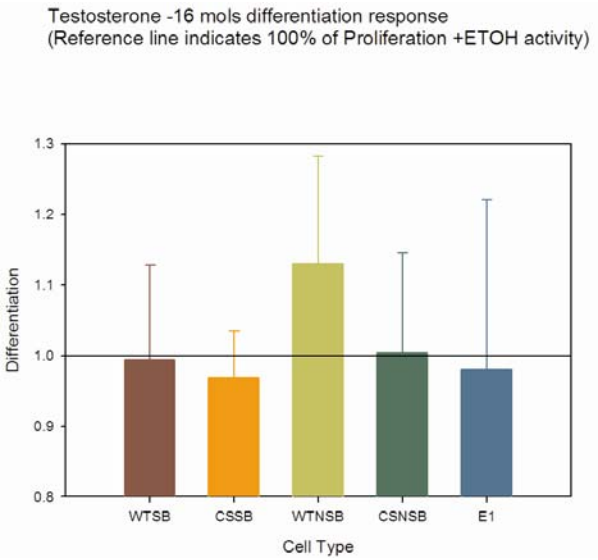

Figure 35: Testosterone -16 mols. Differentiation Activity Normalized to Proliferation Media with Ethanol Response

(1.0 indicates 100% of baseline proliferation media with ethanol added response)

To determine if adding testosterone -16 mols to BMP resulted in altered differentiation activity a paired design was employed comparing proliferation media with BMP added and proliferation with BMP and testosterone added. The assumption of normality was violated for both groups. A natural log transformation was best for normalizing the data, but the testosterone group still violated normality, W=.904, p=.030. Thus, a Wilcoxon paired comparison is used. Proliferation media with BMP added had significantly greater differentiation (χ=.0434, SD

.0408), than proliferation with BMP and testosterone co-culture (χ=0.3781, SD .0374), Z=2.686, p=.007.

The differentiation response of cells treated with testosterone added to BMP normalized to baseline BMP differentiation activity was investigated for difference by phenotype (Wild versus Synostotic) and cell type (suture versus non suture bone) by a two way analysis of variance. **Figure 36** exhibits the means (+/- SE) for each cell type. Wild type suture bone appears to react similarly to BMP baseline levels, and wild type non suture bone appears very inhibited. The assumption of normality was met for each all types. The assumption of homogeneity of variance was met, F=.359, df 3, 20, p=.784. There was no significant difference for phenotype by cell type, F=2.828, df 1, 20, p=.108. There was no significant difference for phenol type averaged across cell type, F=.101, df 1, 20, p=.754. There was a significant difference in differentiation by cell type, suture bone cells (χ=.9540, SD .2313) exhibiting significantly greater differentiation than non suture bone cells (χ=.7440, SD .1528). The non suture cells are thus much more inhibited with testosterone co-culture compared to BMP baseline.

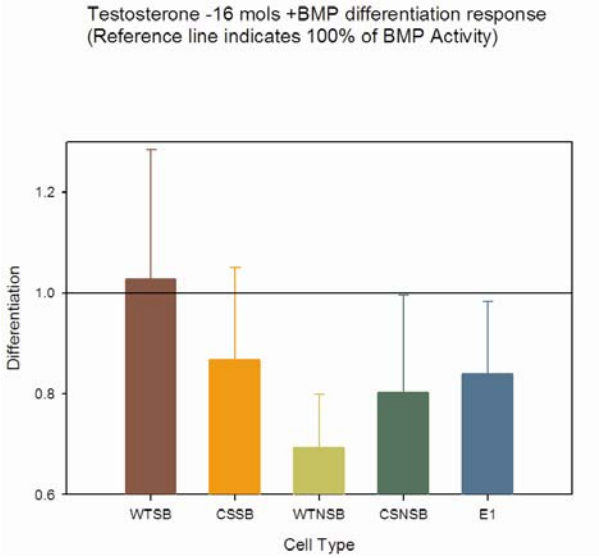

Figure 36: Testosterone -16 mols BMP Co-Culture. Differentiation Activity Normalized to BMP Response

(1.0 indicates 100% of baseline BMP Response)

To determine if adding flutamide resulted in altered differentiation response a paired design was employed comparing proliferation media with ethanol added and proliferation with ethanol and flutamide added. The assumption of normality was violated for both groups. A natural log transformation allowed for normality. Proliferation media with ethanol added exhibited significantly greater differentiation (χ=.0242, SD=.0132) than the flutamide co-culture (χ=.0217, SD .0123), t=3.432, df 47, p=.001.

Differentiation of cells treated with flutamide, normalized to baseline proliferation media with ethanol treatment was investigated for dose (-6, -8, and -10 mols), phenotype (Wild Type versus Synostotic) and cell type (Suture versus Non Suture), by a three way ANOVA. **Figure 37** exhibits the mean (+/- SE) for each cell type. There appears to be two distinct patterns, suture bone decreasing differentiation at lower doses of flutamide, and non suture bone increasing differentiation at lower doses. The assumption of normality was met for all type. The assumption of homogeneity of variance was met, F=2.125, df 11, 36, p=.12. There were no significant

differences in differentiation for phenotype by cell type by dose, F=.062, df 2, 36, p=.940. There were no significant pattern of differences for differentiation by cell type by dose averaged across phenotype, F=.783, df 2, 36, p=.465. There were no significant pattern of differences for differentiation by phenotype by dose, averaged across cell type, F=.025, df 2, 36, p=.975. There were no significant pattern of difference for differentiation by phenotype by cell type averaged across dose, F=1.789, df 1, 36, p=.189. There was no significant main effect for dose, F=.090, df 2, 36, p=.914. There was no significant main effect for cell type, F=.350, df 1, 36, p=558. There was no significant main effect for phenotype, F=.184, df 1, 36, p=.671.

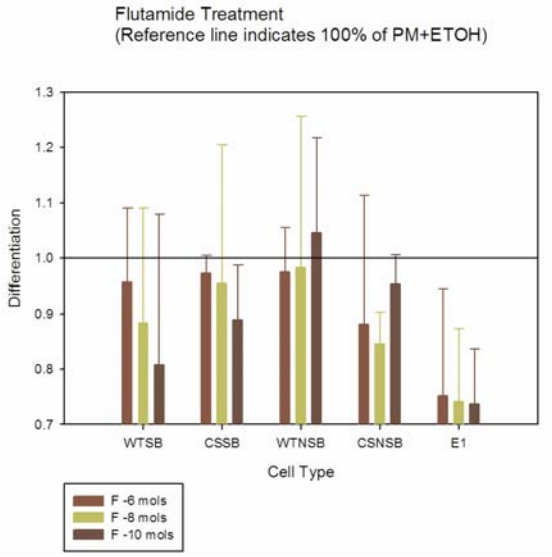

Figure 37: Flutamide Differentiation Activity Normalized to Proliferation Media+Ethanol Response

(1.0 indicates 100% of baseline proliferation media with ethanol added response)

To determine if adding flutamide to BMP resulted in altered differentiation a paired design was employed comparing proliferation media with ethanol and flutamide added to BMP, with proliferation media with BMP added only. The assumption of normality was violated for each group. A natural log transformation allowed for normality. There was no significant

difference in differentiation between proliferation media with BMP added (χ=.0434, SD .0402) and that co-cultured with flutamide (χ=.0453, SD .0449), t=1.123, df 47, p=.267.

Differentiation of cells treated with flutamide and BMP co-culture, normalized to baseline BMP activity, was investigated for dose response (-6, -8, and -10 mols), phenotype (wild type versus synostotic) and cell type (suture versus non suture bone), by three way ANOVA. **Figure 38** exhibits means (+/- SE) for each type. Like flutamide treatment alone there appears to be two distinct patterns, suture bone decreasing differentiation at lower doses of flutamide, and non suture bone increasing differentiation at lower doses. There were multiple violations of normality, synostotic suture bone treated with -10 flutamide, synostotic non suture bone with -6 flutamide, wild type suture bone treated with -6 and -8 flutamide. An inverse transformation worked best, but still had two violations of normality, Synostotic suture bone treated with -10 flutamide, W=.755, P=.012, and Wild type suture bone treated at -6 flutamide, W=.753, p=.006. ANOVA should be robust against these violations. The assumption of homogeneity of variance was met, F=1.670, df 11, 36, p=.121. There was no significant pattern of difference for differentiation by phenotype by cell type by dose, F=2.984, df 2, 36, p=.063. There were no significant pattern of differences by cell type by dose averaged across phenotype, F=1.131, df 2, 36, p=.334. There were no significant pattern of differences by phenotype by dose averaged across cell type, F=.256, df 2, 36, p=.776. There were no significant pattern of differences by phenotype by cell type, averaged across dose, F=2.425, df 1, 36, p=.128. There was no significant dose main effect, F=2.013, df 2, 36, p=.148. There was no significant phenotype main effect, F=.706, df 1, 36, p=.406. There was a significant main effect by cell type, suture bone (χ=1.064, SD .2281) having significantly greater percent differentiation than non suture bone (χ=.9037, SD=.2609), F=.6447, df 1, 36, p=.016.

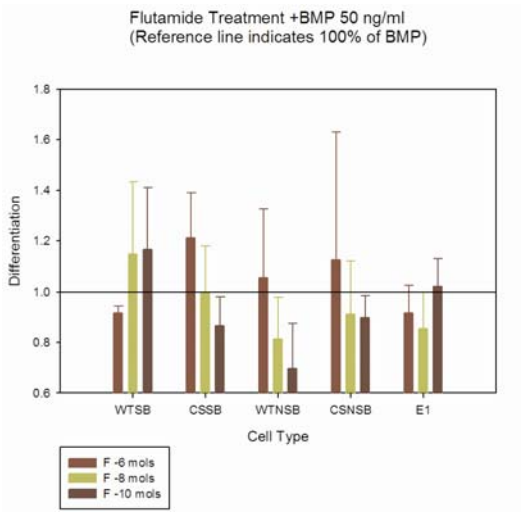

Figure 38: Flutamide + BMP Differentiation Activity Normalized to BMP only response

(1.0 indicates 100% of baseline BMP Response)

To determine if there is an additive effect in reducing differentiation with the addition of flutamide to testosterone in ethanol a paired design was employed comparing the amount of reduction of activity for testosterone and testosterone flutamide co-culture. The assumption of normality was met for both groups. There was a significant difference in percent differentiation activity change, testosterone alone increasing differentiation (χ= -.0236, SD .1339), flutamide co-culture resulting in a significant percent decrease (χ=.1602, SD .1960), t=6.828, df 47, p<.001.

The percent of differentiation reduced by flutamide co-cultured with testosterone compared to testosterone only treatments were investigated for dose (-6, -8, and -10 mols), phenotype (wild versus synostotic) and cell type (suture versus non suture bone) by a three way ANOVA. **Figure 39** exhibits means (+/- SE) for each type. There appears to be several patterns of response, the wild type suture bone exhibiting an increase in reduction of activity as dose of flutamide decreased. Wild type non suture bone demonstrates a biphasic response. Synostotic suture none exhibits a decrease in reduction of differentiation with lower doses, and synostotic

non suture bone appears to show the opposite pattern. The assumption of normality was met for all types. The assumption of homogeneity of variance was met, F=.945, df 11, 36, p=.511. There was no significant pattern of differences by phenotype by cell type by dose, F=1.991, df 2, 36, p=.151. There was no significant pattern of differences by cell type by dose averaged across phenotype, F=.189, df 2, 36, p=.828. There was no significant pattern of differences for phenotype by dose averaged across cell type, F=1.535, df 2, 36, p=.229. There was no significant pattern of difference by phenotype by cell type averaged across dose, F=1.866, df 1, 36, p=.180. There was no significant dose main effect, F=.582, df 2, 36, p=.564. There was no significant cell type main effect, F=.430, df 1, 36, p=.516. There was no significant phenotype main effect, F=.961, df 1, 36, p= .333.

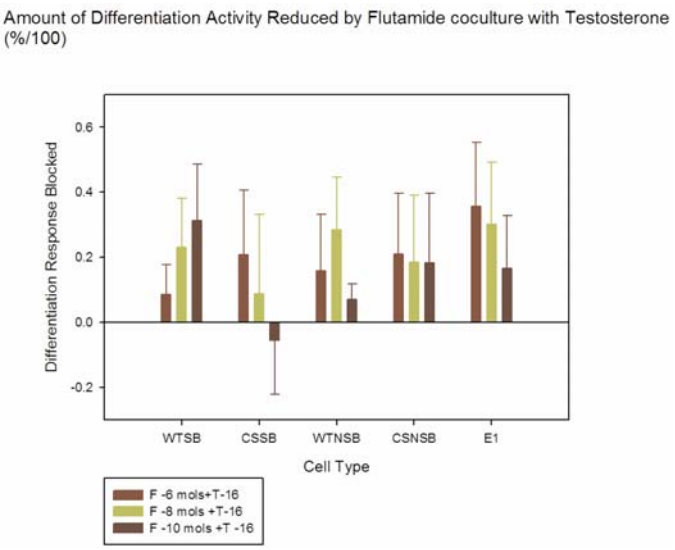

Figure 39: Amount of Differentiation Activity Reduced Flutamide + Testosterone -16 mols.

Differentiation Activity Normalized to Testosterone -16 mols. only response

To determine if there is an additive effect in reducing differentiation with the addition of flutamide to testosterone+BMP a paired design was employed comparing the amount of

reduction of activity for testosterone+BMP and testosterone flutamide BMP co-culture. The assumption of normality was violated for both groups. A square root transformation worked best for normalizing the data but the flutamide co culture remained non-normal, W=.914, p=.011. Thus, a Wilcoxon paired comparison was utilized. The flutamide co-culture (χ=.2798, SD .2127) exhibited significantly greater percent reduction in differentiation compared to testosterone+BMP co-culture (χ=.1422, SD .2193), Z=4.011, p<.001.

The percent reduction of differentiation activity reduced by flutamide co-cultured with testosterone and BMP compared to testosterone and BMP only treatments were investigated for dose (-6, -8, -10), phenotype (wild type versus synostotic), and cell type (suture bone versus non suture bone), by a three way ANOVA. **Figure 40** exhibits the means for each cell type (+/- SE). All types except wild type non suture bone which appears to have a biphasic response appear to increase in reduction of differentiation with decreased dose. The assumption of normality was met for all types. The assumption of homogeneity of variance was met, F=1.804, df 11, 36, p=.100. There were no significant differences in percent differentiation reduction by phenotype by cell type, by dose, F=.844, df 2, 36, p=.438. There was no significant pattern of difference by cell type by dose averaged across phenotype, F=.227, df 2, 36, p=.798. There was no significant pattern of differences by phenotype by dose averaged across cell type, F=.155, df 2, 36, p=.857. There was no significant pattern of differences by phenotype by cell type averaged across dose, F=.001, df 1, 36, p=.973. There was no significant dose main effect, F=1.183, df 2, 36, p=.318. There was no significant cell type main effect, F=.164, df 1, 36, p=.688. There was no significant phenotype main effect, F=.003, df 1, 36, p=.959.

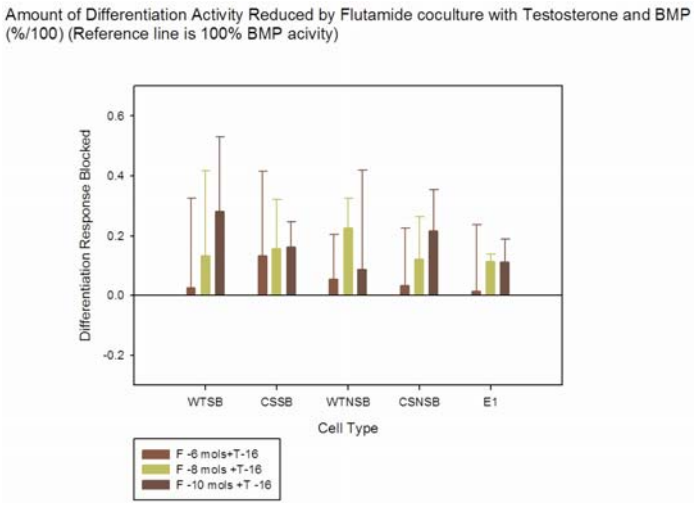

Figure 40: Amount of Differentiation Activity Reduced Flutamide + Testosterone -16 mols BMP Co-Culture

Differentiation Activity Normalized to Testosterone -16 mols. BMP Co-Culture Response

3.3 CULLED CELLULAR DATA

3.3.1 Assessment of Proliferation

To best describe the relationship between bone cell proliferation response when treated with bone morphogenetic protein and testosterone all data was culled, collapsing wild and synostotic phenotypes, and suture and non suture bone, and hormonal treatment levels, in an a priori fashion. Sample sizes, means, standard errors, and standard deviations are listed in **Table 5**. **Figure 41** exhibits the means (+/- SE) for bone cell proliferative response to the various previously described treatments. The greatest proliferative responses appear to be for proliferation media, flutamide treatment, flutamide BMP co-culture and testosterone and

flutamide co-culture. Inhibition of proliferative response appear to be greatest for testosterone treatment and testosterone and BMP co-culture, with less proliferative response also seen compared to other groups for testosterone, flutamide and BMP co-culture. T-tests were used to investigate the following comparison for proliferative response, 1) ethanol vs. testosterone; 2) ethanol vs. flutamide; 3) BMP vs. proliferation media; 4) BMP + testosterone vs. BMP; 5) BMP + flutamide vs. BMP; 6) BMP + testosterone +flutamide vs. BMP; 7) BMP+ testosterone vs. BMP+ testosterone+ flutamide; 8) testosterone vs. testosterone +flutamide; 9) flutamide vs. testosterone +flutamide.

Table 5: Proliferative Response: Culled Data Set Descriptives

Treatment	N	χ	SE	SD
Proliferation Media	42	.7286	.0530	.3437
Ethanol	42	.6918	.0522	.3384
BMP	42	.7090	.0489	.3166
Testosterone	132	.5811	.0220	.2531
Testosterone+BMP	132	.6298	.0189	.2167
Flutamide	48	.7533	.0489	.3389
Flutamide+BMP	48	.7589	.0444	.3074
Testosterone+Flutamide	48	.7369	.0393	.2723
Testosterone+Flutamide+BMP	48	.6930	.0395	.2738

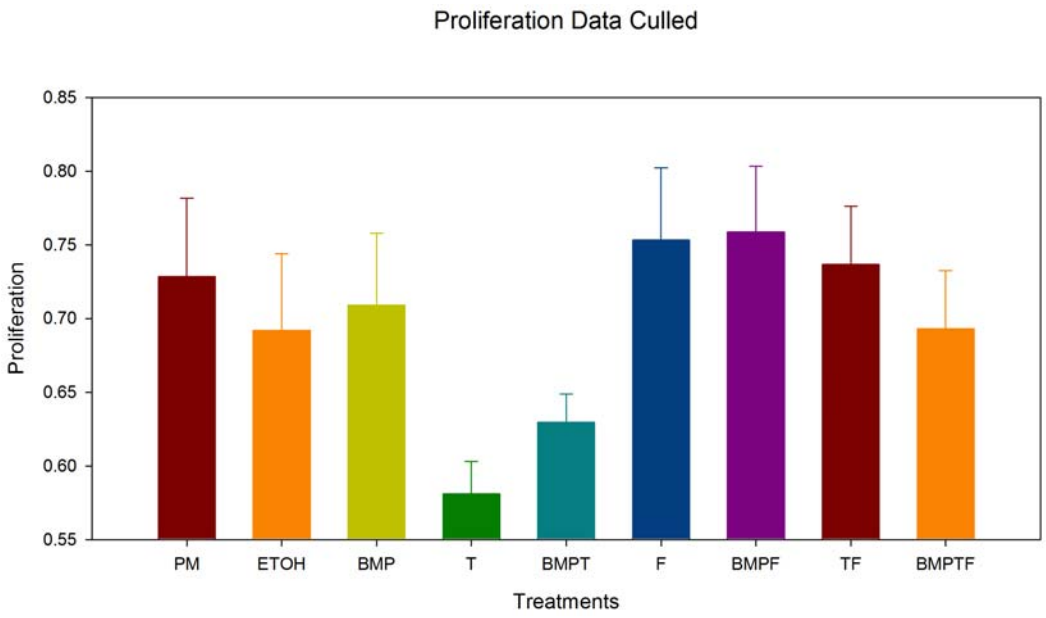

Figure 41: Proliferation Response Culled, Means +/- SE

1) Testosterone appears to significantly inhibit proliferation activity compared to ethanol baseline, t=2.265, df 172, p=.025. 2) Flutamide does not appear to significantly inhibit proliferative activity compared to ethanol baseline, t=.8595, df=88, p=.392. 3) BMP does not appear to differ significantly from baseline proliferation media proliferative activity, t=.2718, df 82, p=.787. 4) BMP + testosterone co-culture does not appear to significantly reduce proliferative activity compared to BMP baseline, t=1.832, df 172, p= 0.069. 5) BMP + flutamide co-culture does not differ significantly in proliferative activity compared to BMP baseline, t=.7569, df 88, p=.451. 6) BMP+ flutamide +testosterone co-culture does not differ significantly in proliferative activity compared to BMP baseline, t=.2571, df 88, p=.798. 7) BMP + testosterone + flutamide co-culture does not differ significantly than BMP+ testosterone in proliferative activity, t=1.608, df 178, p=.110. 8) Testosterone treatment exhibits significantly less proliferative activity than testosterone +flutamide co-culture, t=3.579, df 178, p<.001. 9)

Flutamide treatment does not differ significantly from flutamide+ testosterone co-culture, t=.2614, df=94, p=.794.

3.3.2 Assessment of Differentiation

To best describe the relationship between bone cell differentiation response when treated with bone morphogenetic protein and testosterone all data was culled, collapsing wild and synostotic phenotypes, and suture and non suture bone, and hormonal treatment levels, in an a priori fashion. Sample sizes, means, standard errors, and standard deviations are listed in **Table 6**. **Figure 42** exhibits the means (+/- SE) for bone cell differentiation response to the various previously described treatments. The greatest differentiation was expressed for BMP co-culture with testosterone, BMP alone, and BMP co-culture with flutamide. The testosterone response appears slightly greater than ethanol baseline, flutamide treatment exhibits less differentiation response, with the lowest response observed for testosterone flutamide co-culture. Finally, the BMP testosterone and flutamide co-culture demonstrates less differentiation than BMP, or BMP with testosterone added, T-tests were used to investigate the following comparison for differentiation response, 1) ethanol vs. testosterone; 2) ethanol vs. flutamide; 3) BMP vs. proliferation media; 4) BMP + testosterone vs. BMP; 5) BMP + flutamide vs. BMP; 6) BMP + testosterone +flutamide vs. BMP; 7) BMP+ testosterone vs. BMP+ testosterone+ flutamide; 8) testosterone vs. testosterone +flutamide; 9) flutamide vs. testosterone +flutamide.

Table 6: Differentiation Response: Culled Data Set Descriptives

Treatment	N	χ	SE	SD
Proliferation Media	42	.0330	.0052	.0336
Ethanol	42	.0273	.0043	.0277
BMP	42	.0480	.0079	.0513
Testosterone	132	.0319	.0028	.0321
Testosterone+BMP	132	.0519	.0053	.0608
Flutamide	48	.0217	.0018	.0123
Flutamide+BMP	48	.0453	.0065	.0449
Testosterone+Flutamide	48	.0195	.0016	.0110
Testosterone+Flutamide+BMP	48	.0288	.0034	.0234

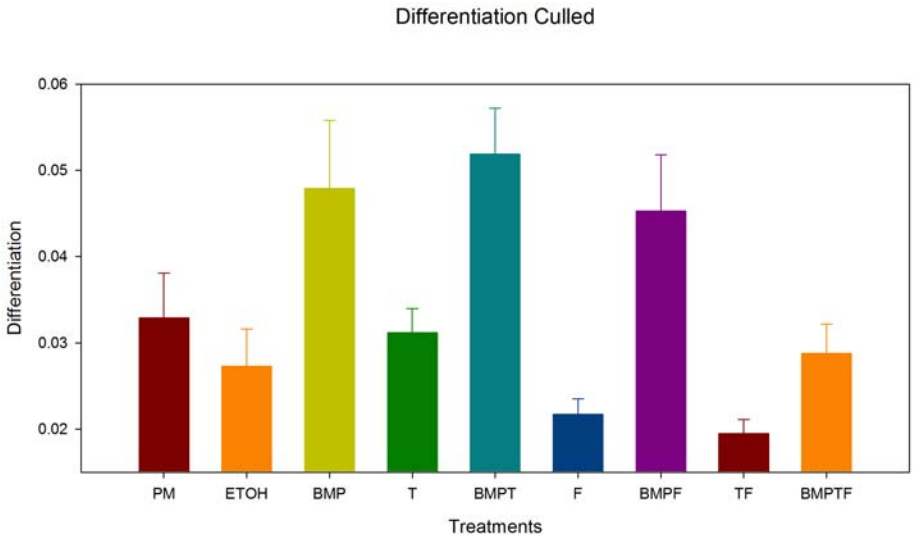

Figure 42: Differentiation Response Culled, Means +/- SE

1) Testosterone does not appear to differ significantly for differentiation compared to ethanol baseline, t=.8451, df 172, p=.400. 2) Flutamide does not appear to differ significantly for differentiation compared to ethanol baseline, t=1.254, df=88, p=.213. 3) BMP did not exhibit significantly different differentiation from baseline proliferation media proliferative activity, t=1.586, df 82, p=.167. 4) BMP + testosterone co-culture does not appear to differ significantly for differentiation compared to BMP baseline, t=.381, df 172, p= 0.704. 5) BMP + flutamide co-culture does not differ significantly for differentiation compared to BMP baseline, t=.2625, df 88, p=.794. 6) BMP+ flutamide +testosterone co-culture exhibited significantly less differentiation compared to BMP baseline, t=2.324, df 88, p=.022. 7) BMP + testosterone + flutamide co-culture exhibited significantly less differentiation compared to BMP+ testosterone, t=2.558, df 178, p=.011. 8) Testosterone treatment exhibits significantly greater differentiation than testosterone +flutamide co-culture, t=2.627, df 178, p=.009. 9) Flutamide treatment does not differ significantly for differentiation than flutamide+ testosterone co-culture, t=.9501, df=94, p=.345.

3.4 ANDROGEN RECEPTOR IDENTIFICATION

3.4.1 Immunohistochemistry

Table 7 provides sample size for animals that tissue was harvested from. Brown coloring would indicate a positive reaction with the antibody. A high concentration of 1:50 primary antibody to 2% serum treatment resulted in no positive staining, **Figure 43**. Thus, the positive

control tissue was negative for the androgen receptor. This indicated that the antibody likely did not recognize the rabbit androgen receptor. Thus, sutures were not stained as even a positive result could not be considered positive.

Table 7: Sample Sizes for Immunohistochemistry Tissue

N	Phenotype	Sex
2	Delayed Onset Synostosis	M/F
2	Early Onset Synostosis	M/F
2	Wild Type	M/F

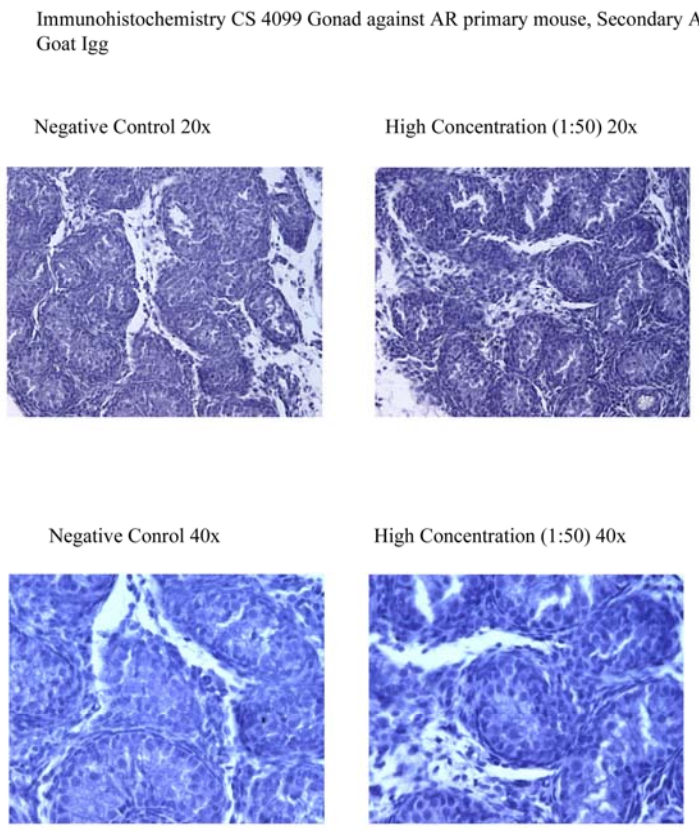

Figure 43: : Immunohistochemistry for Androgen Receptor on Synostotic Testes

Note the Seminiferous Tubules are outlined, and the presence of pale stained Leydig Cells in the Interstitial Areas

3.4.2 Western Blotting

Initial western blotting procedure was for rabbit and MC3T3-E1 cells against the anti-mouse receptor. For the first gel, protein was loaded from left to right with the ladder, MC3T3-E1, wild type suture bone, wild type non suture bone, synostotic suture bone, and synostotic non suture bone at 30 µl per lane for 50ug of protein. Results were negative for all lanes, **Figure 44**. A second run was attempted. Six lanes were loaded in order MC3T3-E1, wild type non suture bone, synostotic suture bone, wild type suture bone, synostotic non suture bone, and synostotic

suture bone. 50 µg of protein was loaded for each well. The results were again negative, **Figure 45**. A positive result was not expected for the MC3T3-E1 lanes, due to incubating with a mouse primary. The rabbit cells were negative allowing for two possibilities, 1) the antibody did not recognize the androgen receptors or 2) the lack of presence of the androgen receptor in these cells.

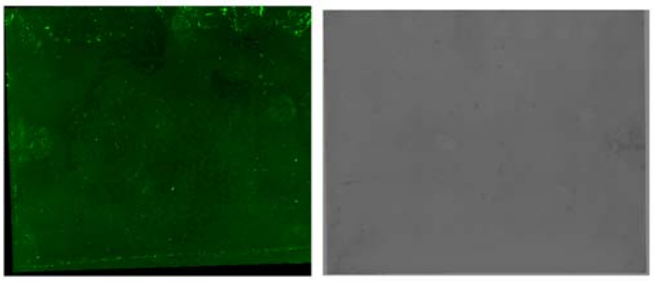

Figure 44: Western Blotting for Androgen Receptors in Rabbit Primary Cells and MC3T3-E1 Immortalized Cells: First Run

Note the Negative Result

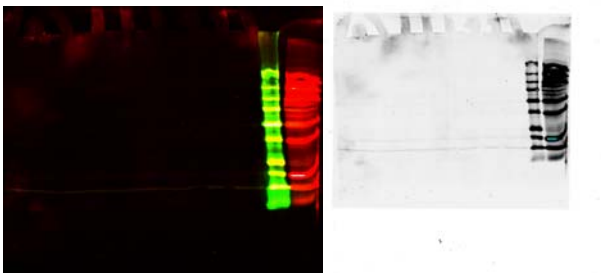

Figure 45: : Western Blotting for Androgen Receptors in Rabbit Primary Cells and MC3T3-E1 Immortalized Cells: Second Run

Note the Negative Result

Western Blotting was conducted for MC3T3-E1 cells against a rabbit polyclonal antibody for androgen receptors and a beta actin control. The cells were loaded in 4 lanes with a total protein density of 10 µg. Results were positive for all lanes for beta actin, **Figure 46**. Results however were negative for androgen receptors. Because this antibody is proven against mouse cells, (Santa Cruz, CA; Stanbrough et al., 2001), it is likely the androgen receptors are not present, or active in these MC3T3-E1 immortalized cells.

Figure 46: : Western Blotting for Androgen Receptors in MC3T3-E1 Immortalized Cells

Note the Negative Result for Androgen Receptors. Beta Actin Control Antibody is Positive

Western Blotting was conducted for rabbit cells against a rabbit polyclonal antibody for androgen receptors. The cells were loaded in 4 lanes in the following order, wild type suture bone, wild type non suture bone, synostotic suture bone, and synostotic non suture bone. Cells were seeded at a density of 50 µg per well. Results were negative for all lanes for both gels, **Figure 47**. A positive result was not expected for the androgen receptors as the primary antibody was raised in rabbits.

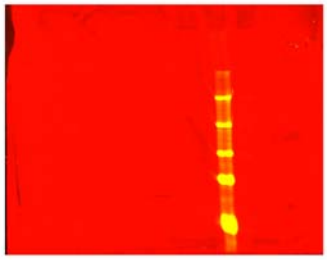

Figure 47: Western Blotting for Androgen Receptors in Rabbit Primary Cells against a Rabbit Polyclonal Antibody

Note the Negative Result for Androgen Receptors.

Western Blotting was conducted for rabbit cells and MC3T3-E1 cells for beta actin, against a primary mouse monoclonal antibody. The cells were loaded onto 2 gels, one for suture bone, wild and synostotic, with MC3T3-E1 controls, and one for non suture bone, wild and synostotic, with MC3T3-E1 controls. For each the cells were loaded in 4 lanes in the following order, MC3T3-E1, synostotic, MC3T3-E1, wild type. Cells were seeded at a density of 25 µg per well. Results were positive for all lanes for beta actin, **Figure 48**.

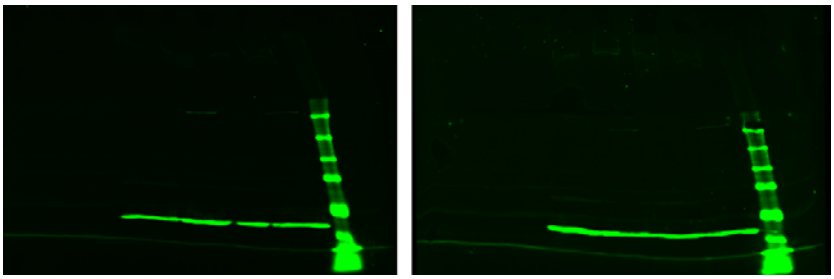

Figure 48: Proof of Principle: Rabbit Primary Cells and MC3T3-E1 Immortalized Cells against a Mouse Monoclonal Beta Actin Antibody

Note the Positive Result for all lanes for both membranes.

Coomasie blue total protein stain was used to identify bands in the 132 kD region of a gel after electrophoresis and membrane after western blotting to determine if protein is present in the area of interest for rabbit cells in lieu of positive immunoflourescent western result. The gel was loaded with wild type suture bone, MC3T3-E1, synostotic suture bone, wild type suture bone, synostotic suture bone, synostotic non suture bone, and reference ladder. 50 µg of protein were loaded per lane. It appears that there are protein bands between the 100-150 kD for each lane, **Figure 49**. Suture bone lanes appeared to stain the darkest, denoting more protein in the area. A second gel was subject to immunoblotting and the resulting membrane was stained with coomasie blue for total protein stain. The lanes were loaded as follows; two reference ladders MC3T3-E1, wild type non suture bone, synostotic suture bone, wild type suture bone, synostotic non-suture bone, and synostotic suture bone. 50 µg of protein were loaded per lane. It appears that there are protein bands between the 100-150 k D for each lane, **Figure 50**.

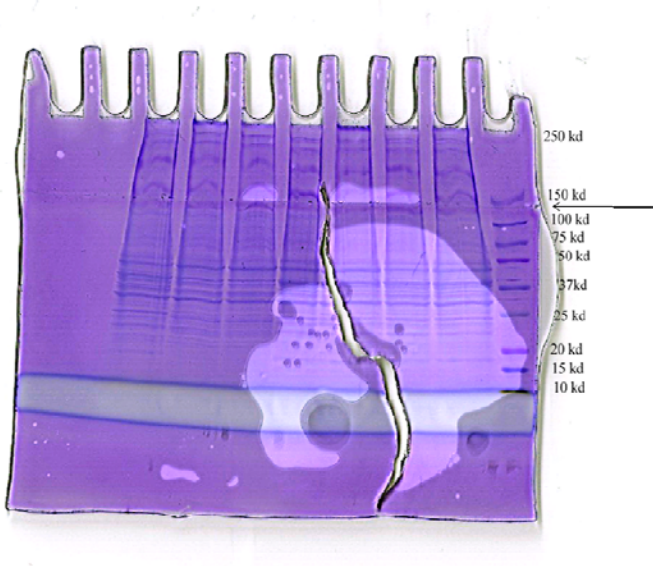

Figure 49: Coomassie Blue Total Protein Stain of Gel after Electrophoresis

Lanes left to right: Wild Type Suture Bone, MC3T3-E1, Synostotic Suture Bone, Wild Type Suture Bone, Synostotic Suture Bone, Synostotic Non Suture Bone, Ladder. Note, tear in gel due to drying.

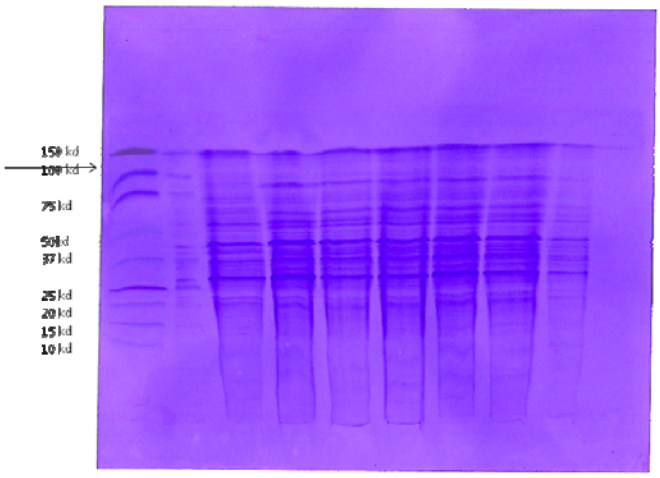

Figure 50: Coomassie Blue Total Protein Stain of Membrane after Immunoblotting

Lanes from left to right- Kaleidoscope Ladder, LiCor Ladder, MC3T3-E1, Wild Type Non Suture Bone, Synostotic Suture Bone, Wild Type Suture Bone, Synostotic Non Suture Bone, Synostotic Suture Bone

3.5 THE EFFECTS OF LOCALIZED FLUTAMIDE THERAPY ON CORONAL SUTURE SYNOSTOSIS AND CRANIOFACIAL GROWTH

3.5.1 Somatic Growth

Table 8 indicates the sample sizes for each group. Body weight was explored for group by age differences. **Figure 51A** exhibits the means (+/- SE) for groups by age. The experimental flutamide group does appear slightly larger at the 25 to 84 day time points, but the slopes appear similar for all groups. The assumption of normality was met for all groups. The assumption of

homogeneity of variance was met for each age, p>.05. There were no significant differences at 10 days of age (F=1.361, p=.278) for body weight. However significant differences for weight by group existed for 25 (F=7.183, p=.004) days, 42 (F=3.066, p=.046) and 84 days of age F=8.441, p=.001. Post hoc least square differences for 25 days of age demonstrates significant differences between the experimental flutamide treatment group (χ=.5200, SD .0932) was significantly larger than the other groups, surgical controls (χ=.3710, SD .1168), p=.005, collagen vehicle controls (χ=.3350, SD .0476), p=.002, and the ethanol control group (χ=.4067, SD .1204), p=.05. This time point occurs before experimental treatment, and thus probably reflects the inherent variability in weight in the colony. Post hoc least square differences for 42 days of age demonstrates significant differences between the experimental flutamide treatment group (χ=1.2638, SD .1965), being significantly larger than surgical controls (χ=1.0190, SD .1472), p=.011, and ethanol treatment controls (χ=1.0080, SD .2476) p=.025. Because there was a pre-existing difference it seems unlikely these weight differences were due to experimental treatment. Post hoc least square differences for 84 days of age demonstrates significant differences between the experimental flutamide treatment group (χ=2.7757, SD .2546), being significantly larger than surgical controls (χ=1.9720, SD .4312), p<.001, and collagen vehicle controls (χ=1.9367, SD .2447), p=.001. Ethanol treatment group (χ=2.5120, SD .5207) also exhibited significantly greater body weights than the surgical controls, p=.016, and collagen vehicle groups, p=.019. Because there was a preexisting difference it seems unlikely these weight differences were due to experimental treatment.

Table 8: Sample Sizes for In Vivo Rescue Study

Group	N
Surgical Control	10
Collagen Vehicle Control	6
Ethanol Control	6
Flutamide Experimental Group	8

Third metacarpal length was explored for differences group by age. **Figure 51B** exhibits the means (+/- SE for each group). The experimental flutamide group appears to diverge at 25 days of age, exhibiting greater metacarpal length, but only at that time point. The collagen vehicle control group appears to exhibit smaller metacarpal lengths at 84 days of age than the other groups. There were multiple violations of normality (ethanol group at 10 days of age, and experimental flutamide group at 10 and 84 days of age). A natural log transformation improved normality, but two violations remained for the experimental flutamide treatment group, 10 days ($W=.728$, $p=.005$) and 84 days ($W=.730$, $p=.013$). ANOVA should be robust against these violations. The assumption of homogeneity of variance met for all ages, $p>.05$. There were no significant differences in third metacarpal length at any time point, 10 day ($F=.829$, $p=.491$), 25 day ($F=1.747$, $p=.185$), 42 day ($F=.521$, $p=.672$) or 84 days of age ($F=1.422$, $p=.275$) by group.

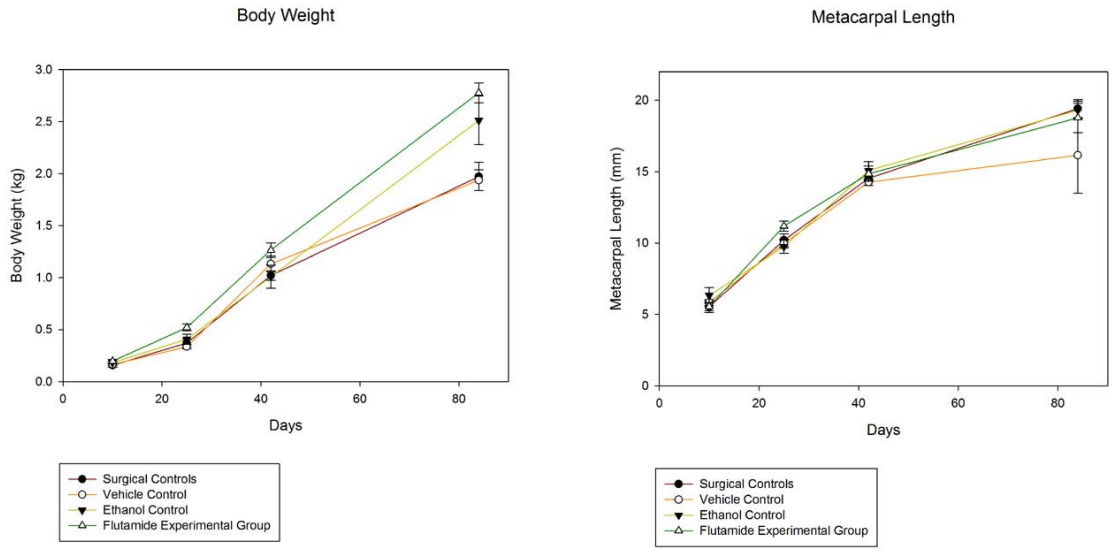

Figure 51: Somatic Growth Comparisons for In Vivo Rescue Study

Legend- A: Bodyweight, B-Metacarpal Length

3.5.2 Cephalometry

Cephalometric landmarks are exhibited in **Figure 12**. **Figure 52** exhibits examples of 84 day lateral cephalographs for each group in the study. Coronal suture marker distance was investigated by one-way ANOVA for each age point. **Figure 53** exhibits the mean growth across the coronal suture by group. At the 25 day time point the surgical control and collagen vehicle control mean separation and are much less than the ethanol control treatment and flutamide experimental treatment, which appear similar. At 42 and 84 days the flutamide group has the greatest growth, followed by the ethanol controls and surgical control group and the collagen vehicle controls exhibiting the least growth across the coronal suture. The assumption of normality was met for all groups. The assumption of homogeneity of variance was met for each

time point, p>.05. There was a significant difference at 25 days of age, F=5.899, df 3, 26, p=.003. Post hoc least square differences exhibit significant differences between the surgical control group (χ=1.1950, SD .5620) and the ethanol control treatment group (χ=2.317, SD .8629), p=.009, as well as the flutamide experimental treatment group (χ=2.1344, SD .9637), p=.016. Significant differences also existed for the collagen vehicle control group (χ=.8417, SD .6778), and the ethanol control group, p=.003, and the flutamide experimental group, p=.004. There was a significant difference in coronal suture marker separation at 42 days, F=2.851, df 3, 24, p=.05. Post hoc least square differences exhibit larger separation for the flutamide experimental group (χ=3.8063 SD .4022) compared to the surgical control group (χ=2.700, SD .5081), p=.029, and the collagen vehicle control group (χ=2.399, SD 1.0842), p=.019. There was no significant difference in coronal suture marker separation at 84 days of age by group, F=1.111, df 3, 25, p=.363.

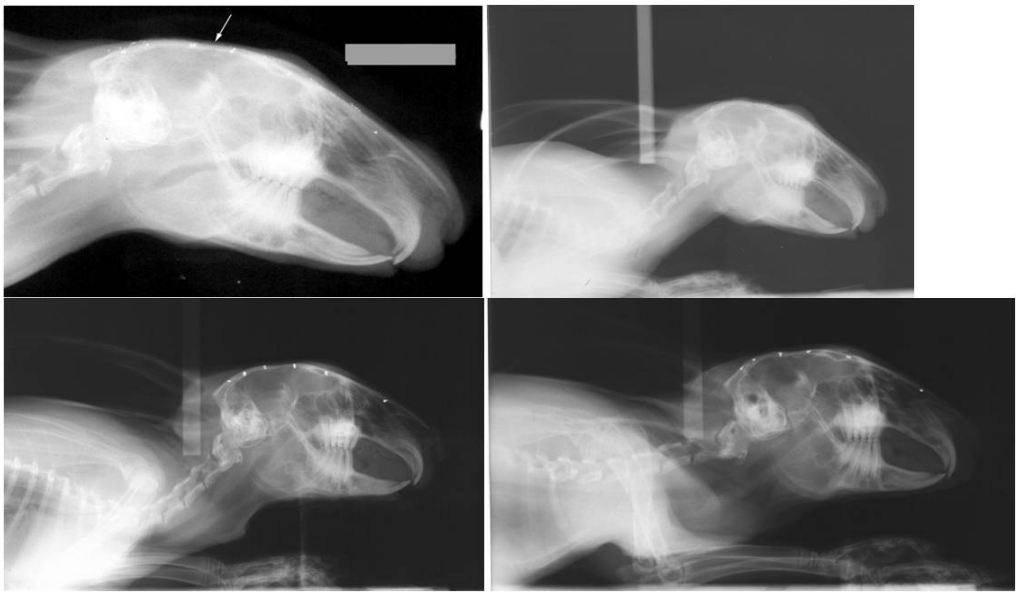

Figure 52: Example of 84 Day Lateral Cephalographs for Each Study Group

Clockwise from Top Left: Surgical Control, BSA Vehicle Control, Ethanol Control, Flutamide Treatment

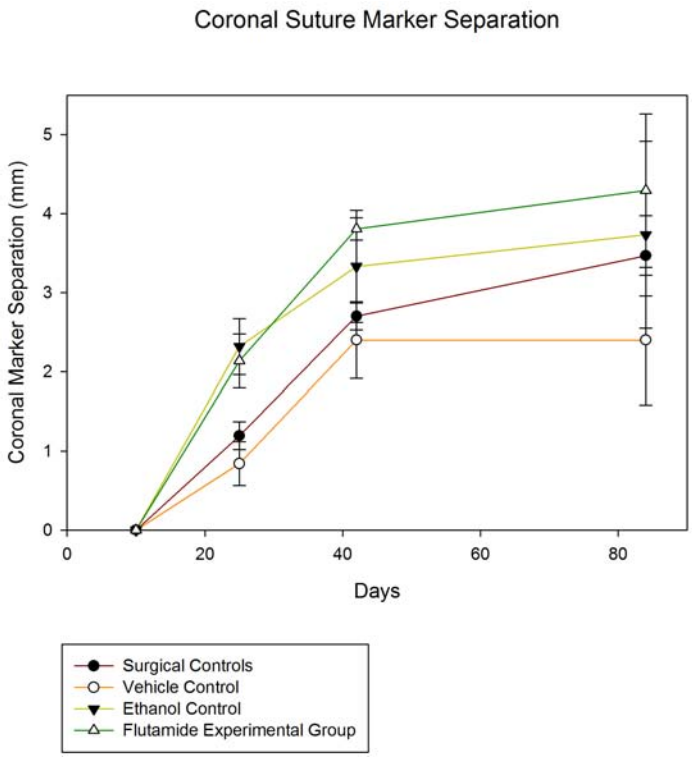

Figure 53: Coronal Suture Marker Separation

Surgical Controls, BSA Vehicle Control, Ethanol Control, and Flutamide Experimental Control

Note the Divergence of the Flutamide group from the Ethanol Controls Between 25 and 42 Days

Craniofacial length was investigated for differences group by age. **Figure 54A** exhibits the mean (+/- SE). All groups demonstrate similar means and growth curves. The assumption of normality was met for all comparisons. The assumption of homogeneity of variance was met for each comparison, $p>.05$ There were no significant differences in craniofacial length by group at 10 days of age, $F=.772$, df 3, 24, $p=.521$, 25 days of age, $F=1.399$, df 3, 25, $p=.266$, 42 days of age, $F=.813$, df 3, 22, $p=.500$, or 84 days of age $F=.991$, df 3, 20 $p=.417$.

An index for intracranial volume was investigated for differences group by age. **Figure 54B** exhibits the mean (+/- SE). All groups start out similarly and retain the similarity from 10-42 days for the means. At 84 days the collagen vehicle group appears to have the highest ICV,

but means do not look too dissimilar. The assumption of normality was met for each comparison. The assumption of homogeneity of variance was met for each comparison. There were significant differences by group for the 10 day, F=3.162, df 3, 25, p=.042, and the 25 day, F=6.831, df 3, 22, p=.002 time points. Least squared significant differences for 10 day data reveal significant differences between the collagen vehicle control group (χ=13365.46, SD 1369.14) and all other groups, surgical controls (χ=11504.95, SD 1600.49), p=.012, ethanol control group (11472.81, SD 1173.62), p=.018, and flutamide experimental group (χ=11639.63, SD 860.68), p=.021. Least squared significant differences for 25 day data reveal significant differences Surgical control group (χ=16031.38, SD=1771.26) and the ethanol control group (χ=18463.95, SD=1962.04), p=.02 and flutamide experimental group (χ=20329.85, SD=2202.14), p<.001. There were no significant differences at 42, F=2.372, df 3, 23, p=.10 and 84 days, F=2.590, df 3, 16, p=.09, for intracranial volume by age. Because the differences occur before experimental intervention, it is likely differences are due to inherent variability in the species.

An index for cranial base length was investigated for differences group by age. **Figure 54C** exhibits the mean (+/- SE). The surgical controls and collagen vehicle controls appear to exhibit a similar growth curve and appear to be greater than the measures for the ethanol control and flutamide experimental group, which appear to have a similar growth curve respectively. The assumption of normality was met for all comparisons. The assumption of homogeneity of variance was met for all comparisons. There were significant differences by group at 10 days of age, F=3.259, df 3, 24, p=.039, 25 days of age, F=3.412, df 3, 25, p=.033, and 84 days of age, F=8.248, df 3, 20, p=.001. There was no significant difference at 42 days of age, F=2.558, df 3, 22 p=.081. The least squared difference for 10 day old data revealed the collagen vehicle control

group (χ=19.46, SD 1.17) to differ significantly from all other groups, surgical control (χ=17.07, SD 1.90), p=.007, ethanol control (χ=17.20, SD 1.15), p=.017, and experimental flutamide treatment (χ=17.56, SD 1.20), p=.031. The least squared difference for 25 day old data revealed the collagen vehicle control group (χ=22.68, SD 1.39) to differ significantly from all other groups, surgical control (χ=20.66, SD 1.66), p=.017, ethanol control (χ=19.98, SD 1.69), p=.005, and experimental flutamide treatment (χ=20.96, SD 0.90), p=.048. The least squared differences for the 84 day old data revealed a dichotomy the surgical control group (χ=27.16, SD 2.03) and collagen vehicle group (χ=29.23, SD 1.28) both differing from the ethanol control group (χ=24.28, SD 0.28) and experimental flutamide treatment (χ=24.87, SD 1.81) respectively (Surgical Control vs. Ethanol Control, p=.011; Surgical Control vs. Experimental Flutamide Treatment, p=.015; Collagen Vehicle Control vs. Ethanol Control, p=.001; Collagen Vehicle Control vs. Experimental Flutamide Treatment, p=.001).

Cranial shape index was investigated for differences group by age. **Figure 54D** exhibits the mean (+/- SE). All groups represent a similar growth curve. There appears to be a derivation for the collagen control group at 42 days of age, it appears to be much greater than the other groups. There were multiple violations of normality. A natural log transformation worked best to normalize the data but two violations remained, collagen vehicle group at 10 days (W=.685, p=.004) and experimental flutamide group at 25 days. ANOVA should be robust against these violations. The assumption of homogeneity of variance was met for all comparisons. There were no significant differences for cranial index by group for any age, 10 days, F=.518, df 3,25, p=.674, 25 days, F=.631, df 3,26, p=.602, 42 days, F=.952, df 3,23, p=.432, or 84 days, F=.195, df 3,21, p=.899.

Cranial length was investigated for differences group by age. **Figure 54E** exhibits the mean (+/-SE). The pattern of growth appears to be the same for all groups. The assumption of normality was violated for two groups, collagen control group at 10 days (W=.743, p=.02) and 42 days (W=.722, p=.02). Standard transformations did not improve on the normality. ANOVA should be robust against this violation. The assumption of homogeneity of variance was met for all comparisons except the 42 day comparison, F=7.634, p=.001. For that comparison a robust test of equality of means, a Welch test, was employed. There were no significant differences in cranial length by group at 10 days of age, F= .404, df 3, 25, p=.751, 25 days, F=1.313, df 3, 26, p=.291, 42 days, F=2.528, df 3, 9.118, p=.122, or 84 days of age, F=.407, df 3, 21, p=.750.

Cranial vault width was investigated for differences by group at each age. **Figure 54F** exhibits the mean (+/-SE). Growth curves appear to be similar with a derivation by the flutamide experimental group at 25 days, appearing to increase in width more than the other groups. The assumption of normality was met for each comparison. The assumption of homogeneity of variance was met for each comparison. There were significant differences by group at 10 days, F= 3.096, df 3, 25, p=.045, and 25 days, F=3.488, df 3, 22 p=.033. Least square differences at 10 days were for the collagen vehicle control group (χ=23.28, SD 1.03) compared with all other groups, surgical controls (χ=21.81, SD 1.42), p=.017 ethanol control (21.60, SD 1.00), p=.013 and flutamide experimental group (χ=21.88, SD 0.68), p=.025. Least square differences at 25 days existed only between the experimental flutamide group (χ=26.58, SD 1.05) and the surgical control group (χ=24.11, SD 2.21), p=.006. There were no significant differences by group at 42 days, F=1.410, df 3, 24, p=.264 or 84 days of age, F=.650, df 3, 17, p=.593.

Cranial height was investigated for differences by group at each age. **Figure 54G** exhibits the mean (+/-SE). The assumption of normality was met for each group. The surgical control

group appears to fall off at 25 and catches up to the other groups by 84 days of age. The ethanol control group appears to have a greater cranial height at 84 days of age. The assumption of homogeneity of variance was met for all comparisons. There were significant differences in cranial height by group for 10 day, F=3.012, df 3, 25, p=.05 and 25 days of age, F=4.182, df 3, 26, p=.015. The least squared differences for 10 days of age demonstrate the collagen vehicle group (χ=18.48, SD .80) to differ significantly from all other groups, surgical controls (χ=16.71, SD .72), p=.011, ethanol controls (χ=16.78, SD .75), p=.029, and flutamide experimental group (χ=16.80, SD .70), p=.022. The least squared difference at 25 days of age exhibited significant differences between the surgical control group (χ=17.96, SD .58) and the ethanol controls (χ=19.25, SD .85), p=.009, as well as the flutamide experimental group (χ=19.23, SD 1.22), p=.006. There were no significant differences for 42 days, F=2.178, df 3, 23, p=.118, or the 84 day comparisons, F=.598, df 3, 21, p=.624.

Cranial base angle was investigated for differences by group at each age. **Figure 54H** exhibits the means (+/-SE). Different patterns of growth appear to exist. The experimental flutamide group shows a decrease in angle over time, the surgical and collagen vehicle controls appear to increase in angle until 42 and then sharply decrease and the ethanol control group appears to have a flat line, or lack of change in angle. The assumption of normality was met for each group. The assumption of homogeneity of variance was met for all comparisons. There were no significant differences in cranial base angle by group at 10 days, F=1.465, df 3, 24, p=.249, 25 days, F=.838, df 3, 25, p=.486, 42 days, F=1.010, df 3, 22, p=.407, or 84 days of age, F=.416, df 3, 20, p=.744.

Palatal angle was investigated for differences by group at each age. **Figure 54I** exhibits the means (+/- SE). A similar pattern of growth exists for all groups, decreasing angle over time,

except for the collagen vehicle control group which appears to exhibit a sharp increase between 10 and 25 days, and tapers off after. The assumption of normality was met for all groups. The assumption of homogeneity of variance was met for each comparison. There was a significant difference in palatal angle by group at 10 days of age, $F=10.247$, df 3, 24, $p<.001$. Least squared significant difference reveal the collagen vehicle control group ($\chi=126.06$ SD 2.98) to exhibit a less obtuse angle than every group, surgical controls ($\chi=130.91$, SD 2.43), $p=.001$, ethanol control ($\chi=132.58$ SD 2.82), $p<.001$, and flutamide experimental group ($\chi=133.03$, SD 1.20), $p<.001$. There were no significant differences in palatal angle by group at 25 days, $F=1.269$, df 3, 25, $p=.306$, 42 days, $F=2.888$, df 3, 22, $p=.06$, or 84 days of age, $F=.569$, df 3, 20, $p=.642$.

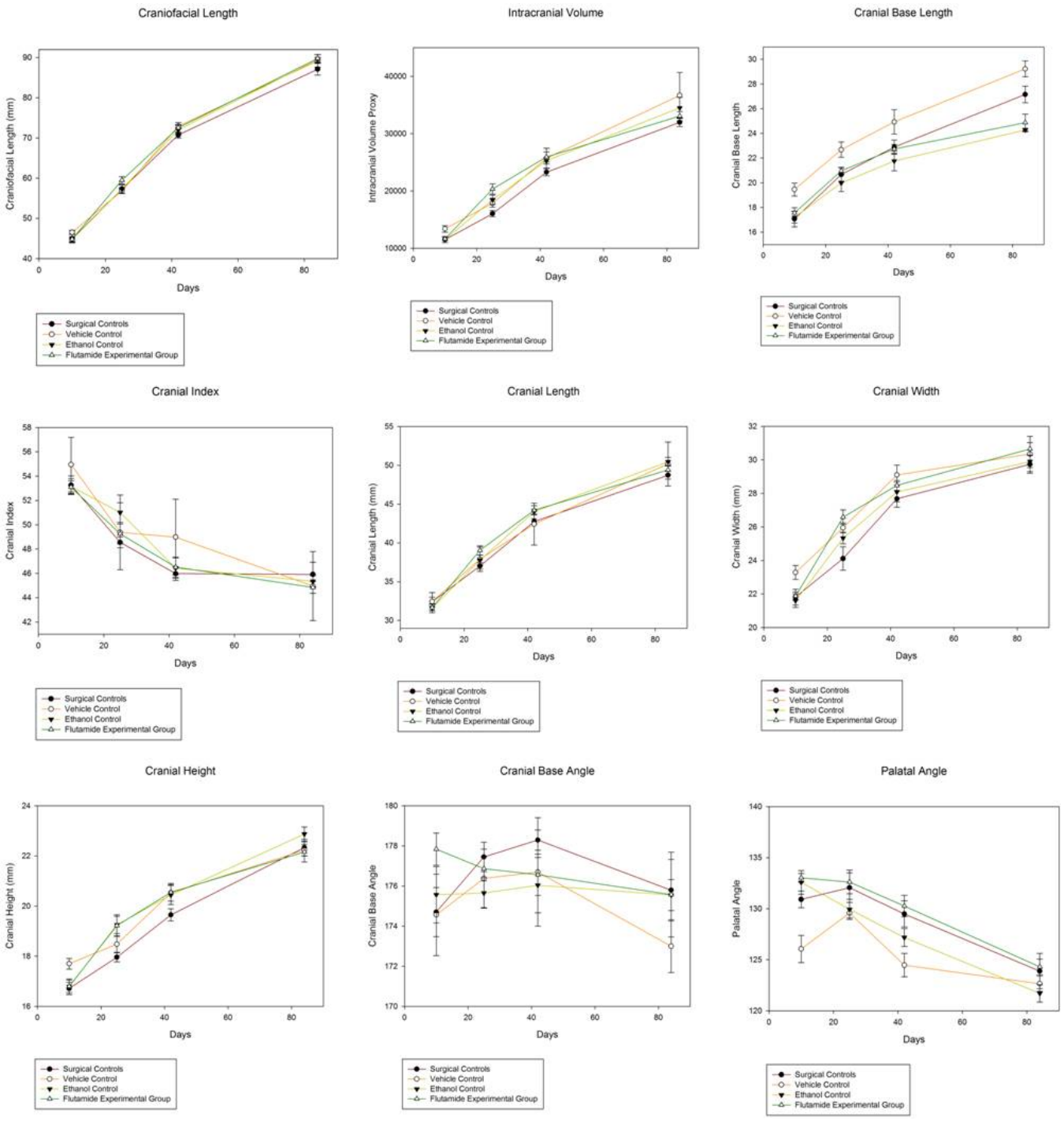

Figure 54: Cephalometry

Surgical Controls, BSA Vehicle Control, Ethanol Control, and Flutamide Experimental Control

Left to Right, Top to Bottom: A. Craniofacial Length, B. Intracranial Volume, C. Cranial Base Length, D. Cranial Shape Index, E. Cranial Length, F. Cranial Width, G. Cranial Height, H. Cranial Base Angle, I. Palatal Angle

4.0 DISCUSSION

4.1 MAJOR CELLULAR MORPHOLOGY RESULTS

4.1.1 Overview

The primary goal of the characterization of cellular response to testosterone was to determine if differences existed between our craniosynostotic rabbit colony and wild type controls. This investigation was specific to osteoblast activity, proliferation and differentiation. Increased susceptibility to testosterone exposure may be a possible pathway for the increase in bone seen at the synostotic rabbit coronal suture. An interesting by-product of these analyses was the suture bone versus non-suture bone comparison for these same effects.

Cells treated with ethanol showed decreased proliferation and differentiation compared to baseline, irrespective of phenotype or bone type. The effects of ethanol on bone cells were tested because water and PBS were not sufficient to reconstitute the flutamide. For each assay in this study, MC3T3-E1 cells were used as an established positive control. MC3T3-E1 cells were found to have low variability within experiments, suggesting consistent assay techniques.

Because it stimulates osteogenic differentiation, BMP administration was expected to reduce cell proliferation. However, both suture and non-suture bone of our affected synostotic rabbits showed an increase in proliferation at 7 days after BMP treatment, compared to baseline.

The standard error bars are sizeable. This error reflected the true variability in these samples. Further, these data may point toward a greater variation in cellular response than reported in the literature. Here, the sample N equaled the number rabbits used for the study, not the number of wells run, or times the same cells were run. To reiterate, three wells were used for each study and averaged to compute a mean for the plate. The grand mean resulted from averaging three identical plates. The wild type suture bone was also comparable to baseline numbers. A true decrease in proliferative activity after BMP treatment may occur earlier with recovery by seven days. However, pretest proliferation analyses allowed for determination of both time and cell density for cell culture. Thus, for these cells, BMP did not decrease proliferation as expected.

Although not significant, testosterone treatment did seem to effect proliferation averaged across cell types. The data suggest testosterone increases proliferation. The dose response curve demonstrates an interesting trend. Synostotic cells had substantially increased proliferation response with testosterone treatments above -30mol. There also appeared to be a biphasic response to testosterone for rabbit cells with peak reactions around -12 and -30mol treatments. The synostotic suture bone at -12 and -30mol treatments was more proliferative than other cell types. The non-suture bone also showing an increase in activity at -30 mols. However, analysis of variance did not show differences by dose, bone type, phenotype, or any interaction, suggesting presence of exogenous testosterone might affect proliferation for bone cells independent of these doses. Though the doses may appear to be unnecessarily low, increased proliferation was demonstrated at the -30mol dose for synostotic suture bone.

For all cell types, combined testosterone and BMP treatment did not significantly alter proliferation compared to BMP treatment alone. However, the synostotic suture bone cells exhibit between 1.4 and 1.6 times the proliferative activity compared to baseline values.

Synostotic non-suture bone nearly doubled its proliferation at -16mol compared to baseline. Lower concentrations of testosterone had less effect. Other cell types treated with both testosterone and BMP show little change in proliferation compared to BMP treatment. Non-suture bone showed peak proliferation at -16mol while suture bone peaked at -14 mols, suggesting regional differences in proliferative response to combined BMP and testosterone treatment. However, there were no statistically significant differences for dosage, bone type, phenotype, or interaction terms. Thus, these data suggest treatment with exogenous testosterone alone or with BMP does not significantly affect proliferation in calvarial bone cells.

There was precedent for collapsing cell type, phenotype, and dose data sets to more generally compare groups because none were strong predictors of proliferative response. The resulting N is then 132. This analysis showed that testosterone treatment decreased proliferation. Though testosterone and BMP co-culture also decreased proliferation compared to BMP alone, the differences were not statistically significant. Thus, testosterone does appear to inhibit proliferation alone and in co-culture with BMP, suggesting the cells may be differentiating or calcifying.

BMP treatment appears to affect differentiation. Synostotic non-suture bone exhibited a greater response to BMP stimulation compared to suture bone. This result was opposite of the hypothesis that bone derived from a previous growth site would have more differentiation response than bone derived from a non-growth site. When ALP activity was averaged across cell types, BMP did not significantly alter differentiation. However, the p value was extremely close to significance, suggesting a trend toward increased differentiation. The collapsed data also shows a statistically insignificant increase in differentiation caused by BMP treatment.

Testosterone administration elicits greater differentiation in the synostotic cells compared to wild type controls. Within synostotic cells, the non-suture bone exhibited more differentiation activity than suture bone. The non-suture bone exhibited a biphasic dose response curve with peak differentiation activity at -16 mols. and -30 mols. treatments. The -30 mols. dose exhibited a 20x increase in differentiation activity. Synostotic suture bone exhibited the greatest differentiation at -14 mols, and -30mol, suggesting a biphasic response as well. Other cell types did not significantly differ from baseline. These measurements taken from these experiments showed high levels of variation between subjects (rabbits). These data, averaged across cell types, suggest testosterone treatment did not significantly alter differentiation. Wild-type animals exhibited greater differentiation under testosterone administration for suture bone compared non-suture bone. Synostotic affected rabbit cells exhibited the opposite relationship. In addition, the synostotic cells from suture and non-suture bone, exhibited greater differentiation than wild type cells. Again, dose did not show statistically significant differences. The collapsed data demonstrated testosterone administration significantly inhibited differentiation. Contra most reports in the literature, testosterone administration decreased both proliferation and differentiation. This point will be further discussed below (section 4.1.2).

For all cell types combined, testosterone and BMP co-culture treatment significantly increased the differentiation response compared to BMP treatment alone. This result suggests an additive relationship for BMP with testosterone. Synostotic non-suture cells had peak differentiation activity at -14 mols. testosterone with BMP treatment. The -14 mols. dose resulted in a mean 40x increase in differentiation compared to BMP only treatment. Synostotic suture bone had peak differentiation at a lower dose, -16 mols. The wild type cells do not differ from baseline BMP differentiation measures. For this analysis, synostotic cell types exhibit a large

amount of variation about the mean. This relationship reflects the variation in the synostotic rabbit colony. The analysis of variance suggested no significant differences by dose, phenotype, or interaction terms. However, non-suture bone does have greater differentiation measures compared to suture bone for testosterone and BMP treatment. The collapsed data set demonstrated that testosterone BMP co-culture resulted in an increase in differentiation compared to BMP baseline. Thus, testosterone did increase the effects of BMP on osteoblast differentiation.

The primary goal of the characterization of cellular response to flutamide was to determine if the effects noted in the testosterone administration data could be mediated using this androgen receptor blocker. The expectation was that flutamide administration alone would not significantly affect proliferation or differentiation. When not in the presence of androgen flutamide should have little effect. However, in the presence of testosterone effects should be noted.

For these characterizations, a constant -16 mols. testosterone dose was chosen. This concentration coincided with the greatest differentiation response for testosterone with BMP added for the testosterone studies. Control data showed BMP decreased proliferation compared to control baseline values. In addition, testosterone showed no differences or proliferation by dose, cell type, phenotype, or significant interaction terms, consistent with the above testosterone analyses. Combined testosterone and BMP treatments significantly decreased proliferative activity when compared to BMP baseline. There were no differences, however, for cell type, phenotype, or interaction terms. The collapsed data set analysis was consistent with these findings.

For all cell types, flutamide treatment significantly reduced proliferation activity compared to baseline proliferative values. This result may indicate the presence of endogenous receptors in these cells. However, there were no significant differences by doses, cell type, phenotype, or significant interaction. This suggests the cell types have equal response to flutamide administration. The collapsed data did not show a significant difference between flutamide and baseline values. This suggests the effects of flutamide administration on cell proliferation are minimal.

Combined flutamide and BMP administration significantly decreased proliferation compared to BMP baseline values. Flutamide appeared to have the greatest effect, inhibition, in the wild type cells. There was a dose dependent response across all cell types. Cell treated with flutamide at -10 mol. dose treatments showed less proliferative activity than other doses. This result suggests flutamide is similar to testosterone in that it can demonstrate a biphasic response for proliferation. However, the collapsed data did not exhibit a difference for proliferation after combined flutamide and BMP treatment. This observation provides further evidence that there is great cell response variation inherent in these samples.

Flutamide administration was expected to have the greatest effect in the presence of testosterone. Data above suggest testosterone inhibited cell proliferation in these rabbit cells. For all cell types, combined flutamide and testosterone treatment did not significantly alter proliferation compared to testosterone treatment alone. The flutamide response was variable, but most permutations showed a decrease in proliferation. However, synostotic suture bone at -10 mols and wild type cells from non-suture bone at -8 mols showed an increase in proliferation. These results may suggest some difference in responsiveness by dose and cell type. Nevertheless, the collapsed data set demonstrated a significant increase in cell proliferation for

combined testosterone and flutamide treatment compared to testosterone treatment alone. This result provides evidence that flutamide mediates the effects of testosterone for cell proliferation.

The combined flutamide, BMP, and testosterone co-culture treatment significantly reduced proliferation activity compared to combined BMP with testosterone treatment. This effect was especially strong or wild type non-suture bone. There were no differences found by dose, or phenotype. However, non-suture bone showed a greater reduction in proliferative activity compared to suture bone. Conversely, the collapsed data set did not exhibit a significant difference between combined flutamide, BMP, and testosterone compared to BMP and testosterone treatment alone.

To reiterate for the control data, BMP treatment significantly increased differentiation compared to baseline values. Wild type suture bone had the highest values for differentiation with BMP treatment. However, there were no significant differences by phenotype or cell type. Testosterone treatment did not increase differentiation. Wild type non-suture bone had the greatest differentiation upon testosterone treatment. Again, there were no significant differences by phenotype or cell type. In addition, for combined testosterone and BMP treatment, suture bone had significantly greater differentiation response compared to non-suture bone. Combined testosterone and BMP treatment decreased differentiation for this -16 mols. dose. The results from the testosterone study are not consistent with these results where the co-culture demonstrated increased differentiation. This result reflects the inherent variability between the animals utilized.

For all cell types, flutamide treatment decreased differentiation from baseline values. Again, it is possible that flutamide was targeting endogenous testosterone and/or androgen receptors present in the cells. A greater effect on differentiation, decrease, at lower doses of

flutamide was noted for suture bone. Non-suture bone had decreases in differentiation activity at higher flutamide dose. However, there were no significant differences by phenotype, cell type, or dose for amount of differentiation with flutamide treatment. The collapsed data set showed no significant difference, between flutamide treatment, and baseline values for differentiation. This result suggests flutamide had little effect on these cells when administered alone.

Combined flutamide and BMP treatment did not significantly affect differentiation compared to BMP baseline differentiation. There were not significant differences for dose or phenotype. Suture bone did exhibit a significantly greater increase in differentiation compared to non-suture bone. This result suggests regional differences in susceptibility to flutamide treatment. However, the combined data set shows no significant difference for differentiation between combined BMP and flutamide treatment compared to BMP alone. This result suggests overall, flutamide does not the effect of BMP on osteoblast differentiation.

For all cell types, combined flutamide and testosterone treatment reduced differentiation. However, there were no significant differences by dose, phenotype, or dosage. This result suggests all cell types respond similarly to this combined treatment. The collapsed data indicated showed that combined flutamide and testosterone treatment significantly inhibited differentiation. This treatment resulted in the least amount of differentiation of all treatments.

Combined flutamide, testosterone, and BMP treatment showed a significant decrease of the differentiation response compared to combined testosterone and BMP treatment. All cell types except wild type non-suture bone responded in a dose dependent manner, the higher doses of flutamide resulted in less differentiation. However, there were no significant differences for reduction in differentiation by cell type, phenotype, or dosage, again suggesting a similar

response across all type. The collapsed data analysis showed the same relationship. The flutamide, BMP, and testosterone combined treatment decreased differentiation.

To briefly summarize these cellular results, very few differences existed by phenotype and cell type. In addition, there were no significant differences existed by dose. There was great variation in measures reflecting the biological, cellular, and molecular reality of the study of hormones. Inter-individual variation existed as well. This reflects the actuality of cellular studies using a true sample size. For testosterone and combined testosterone and BMP treatment, the synostotic cell dose response curves were impressive. However, there were no significant differences by dose. This appears to be due to statistical outliers. These outliers point toward a physiological spectrum for sensitivity to exogenous androgenic exposure. The wild type animals did not exhibit this same variation. These results suggest there may be an interaction between androgenic hormone exposure and the overproduction of bone in our affected animals. The outcomes of these studies suggest a little hormone exposure during a critical time window can majorly affect bony growth.

Moreover, it appears that suture bone and non-suture bone are more reactive to an increase in differentiation at different doses. Suture bone peaked in its differentiation activity at a slightly lower dose than non-suture bone, which suggests different biphasic curve responses by bone types. In general, it appeared that suture bone responded less to flutamide, testosterone, and BMP co-culture than non-suture bone.

The collapsed data set provided a clear indicating of these experimental treatment effects on calvarial bone derived cells. Testosterone and combined testosterone and BMP treatment resulted in a decrease in proliferative activity. These results suggest that the cells were no longer proliferating and were differentiating or even constructing a calcified matrix. In addition, it

appeared that flutamide mediated these effects. Flutamide treatment groups resulted in a greater proliferative response. Thus, testosterone appeared to inhibit proliferation, alone and with BMP. This effect was rescued by flutamide administration.

Research suggests BMP should elicit greater osteoblast differentiation. These data show testosterone to increase the effects of BMP on differentiation. The testosterone treatment did not exhibit an increase in differentiation. This result suggests the androgenic effect on osteoblast differentiation can be mediated through the BMP pathway. Flutamide administration greatly decreased the combined testosterone and BMP effect on differentiation. This result suggests that flutamide can counteract the effect that androgenic hormone has on BMP in osteoblast differentiation. As flutamide is a specific androgen receptor blocker, it follows that androgen receptors must have been present in these calvarial derived cells.

4.1.2 Comparison to Studies in the Literature

Unlike reports in the literature (Kasperk et al., 1989), the data here suggest that the effect testosterone has is dependent upon the presence of BMP. Testosterone alone demonstrated little effect on cell proliferation. It may be that other growth factors enhance skeletal growth when treated with testosterone. These growth factors may include the FGFs, IGFs, and TGFβs (Gori et al., 1999; Hofbauer et al., 1998). The MAP kinase ERK-1 can mediate testosterone's proliferative effect. MAP kinase is redundant in most cell proliferation and differentiation pathways (Hofbauer et al., 1998; Wiren et al., 2004). Data from the present study is in contrast to what Kasra and Grynpas (1995) concluded with in vivo study of primate bone densities after long term androgen exposure (Kasra and Grynpas, 1995). It is most likely that the observed differences in bone density were due to the effect that testosterone has on muscle development

and not that it directly affected bone cell biology (Compston, 2001; Notelovitz, 2002; Vanderschueren et al., 2004; Wiren and Orwoll, 2002; Wiren, 2005). Additionally, bone marrow has also been the target of investigation for a testosterone dependent effect, It appeared that testosterone increased endothelial cell proliferation, which suggests a possible mechanism for maintenance of bone integrity in general (Foresta et al., 2008; Ray et al., 2008). In addition, testosterone also decreased the proliferation of osteoclasts (Michael et al., 2005).

Wang et al. (2007), using DHEA on a primary murine osteoblast cell line showed an increase proliferation and decrease apoptosis via the MAP kinase pathway (**Figure 55).** This demonstrated the effectiveness of an adrenal based hormone to mediate bone cell morphology, independent of androgen or estrogen receptors (Wang et al., 2007). Interestingly, BMP-2 has also been implicated as an agonist to the MAP kinase ERK pathway, increasing phosphorlyation of SMAD and increasing the expression of P38 and ERK downstream (Ghosh-Choudhury et al., 2007; Naganawa et al., 2008; Tang et al., 2008). It may also be that dihydrotestosterone induces a phosphorlyation of the protein kinase Akt, which induces translocation to the osteoblast nucleus, causing cell growth (Kang et al., 2004). Miki et al. (2007) also found that an aromatase inhibitor increased osteoblast proliferation in a human osteoblast cell line. This effect was inhibited by flutamide administration suggesting an androgen receptor specific response. However, these authors also found, via reverse transcriptase polymerase chain reaction, an increase in the expression of HOX D11. Thus, androgens can affect the osteoblast lineage via AR dependent or independent pathways (Miki et al., 2007). In addition, it would seem that HOXD 11 expression could be upregulated even if all of its downstream targets were not. Flutamide administration did decrease osteoblast proliferation in Miki et al. study. It is important to note that adrenal androgens appear to have several pathways in which osteoblasts and bone

formation can be affected. However, it is unclear, if the more potent testosterone and dihydrotestosterone both have the same affinity as the adrenal hormones. Since cell data from the present study showed a significant effect with flutamide administration, it was probably mediated by androgen receptors.

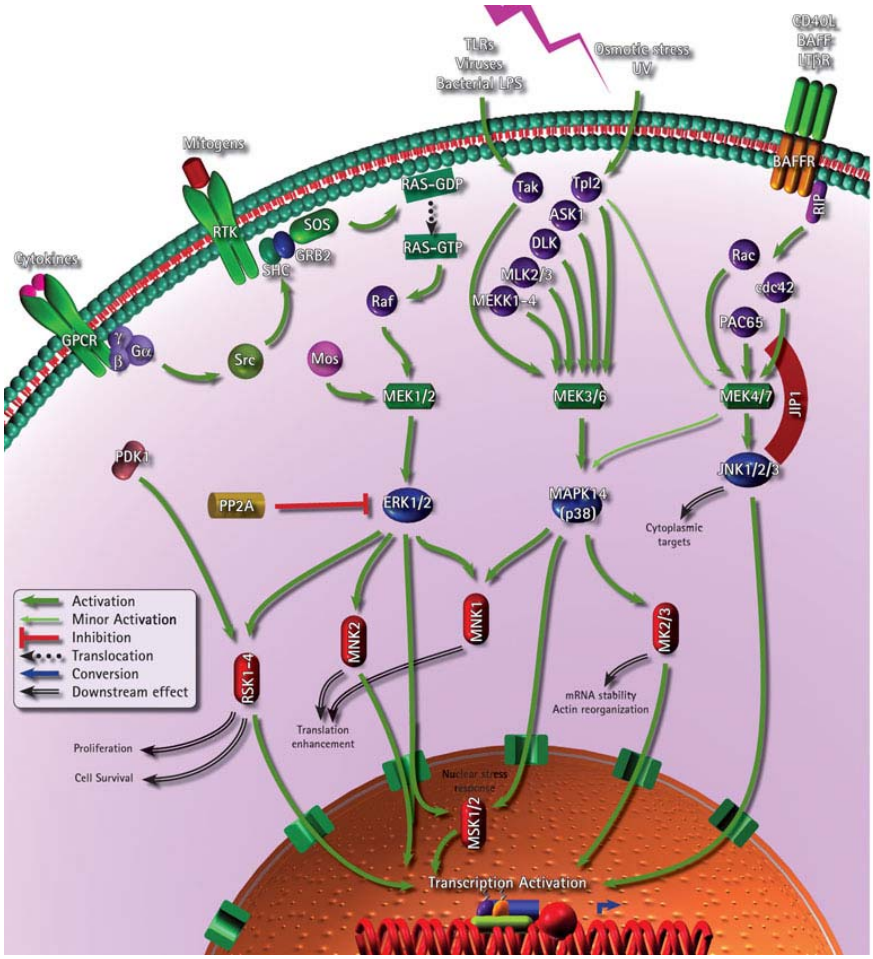

Figure 55: Mitogen Activated Protein Kinase Pathway

Modified from http://www.emdbiosciences.com/popup/CBC/ip_mapk_signaling_pathway.htm

Lin et al. (2007), after androgenic administration in fetal mouse calvarial osteoblasts and dural cells showed increased proliferation and differentiation, suggesting a possible role in suture fusion. This is contra to the results found here and those reported by Chen et al. (2000). Chen et

al. (2000) reported that testosterone administration in a rat fetal calvarial model decreased proliferation, and slightly increased differentiation. The only difference may be that Lin et al (2007) used dihydrotestosterone, a much more potent androgenic hormone than testosterone.

Thus, data from the present study are unique in that they addressed the interaction of testosterone and BMP on osteoblasts. Testosterone appeared to increase BMP's affinity for osteoblast differentiation, this decreasing proliferation. In addition, this primary cell culture model reflected a true sample size with its large spectrum of variation, not generally used in most cell culture studies.

4.2 SUMMARY OF ANDROGEN RECEPTOR FINDINGS AND COMPARISON TO PUBLISHED STUDIES

Androgen receptors in the gonads, calvarial, and suture derived bone cells in study animal could not be identified using Western Blots or immunohistochemistry. This was expected because, the only antibody commercially available was a monoclonal antibody made in mouse against human. These antibodies are made to reduce cross reactivity. It is interesting that the anti-rabbit polyclonal antibody was also not successful in identifying the androgen receptor in mouse-derived. Because gonads are an active site of androgen receptors, the immunohistochemistry should have been positive on these slides. This was good evidence that our antibody would not work on the multitude of suture derived histology slides cut. It was known that using a monoclonal antibody such as this one, would be problematic due to its high level of specificity. Any change in the structure of the epitope would result in a negative result.

Unfortunately, this was the only antibody commercially available for the androgen receptor that was not made in rabbit.

Other researchers have identified the androgen receptor in the calvaria by using immunohistochemistry. Lin et al. (2004), was able to identify androgen receptors in the posterior frontal suture dura, posterior frontal osteogenic front, coronal suture dura, sagittal suture dura, and the sagittal suture osteogenic front of late gestation fetal mice. Interestingly however, Lin et al., (2004) reported no staining (or inconsistent staining) for the coronal suture osteogenic front and the coronal suture midsutural mesenchyme. This discovery laid the basis for the present investigation on the effects of androgens on suture biology and subsequent craniofacial growth.

The western blot analyses were equally as unproductive, mostly because the MC3T3-E1 cells demonstrated no presence of an androgen receptor. The membranes of the western blot were positive for protein transfer as identified by coomassie blue total protein stains, and protein bands did exist for all cell types between 100-150 k Da where the androgen receptor is located. In addition, membranes were positive for beta actin, a cytoskeletal actin gene that is often used to compare intensity of proteins present on immunoblotting and successful transfer of protein, for all cell types. It was assumed that the MC3T3-E1 cells would be positive for the androgen receptors. Upon further inspection into the literature only three studies demonstrated positive results for AR using western blots in MC3T3-E1 cells (Balkan et al., 2005; Nakano et al., 1994; Wiren et al., 2002). These studies pretreated or transfected cells used with androgenic hormone derivates at least three days in culture, prior to analyses. Upon testosterone exposure, androgen receptors translocate to the nucleus from the cell membrane and which when bound to androgen response elements can transactivate target genes (Lin et al., 2004). Thus, the results of the present study suggest that it may be necessary to expose cells to androgenic hormone prior to

western blotting to allow for identification of the androgen receptor. The methodological basis behind this practice is not clear, as all cells are lysed during processing for western blotting, releasing the nucleus. In addition, no published studies have identified androgen receptors in the bone cells of rabbit, and as stated above there is no commercially available antibody that is proper for use in rabbit for identification of the androgen receptor. Finally, as an aside, it seems unlikely that there would have been differences in cellular response, proliferation and differentiation, to testosterone, and that the cellular responses could have been mediated with flutamide treatment, if androgen receptors were not present in these cells.

4.3 IN VIVO FLUTAMIDE THERAPY

4.3.1 Overview

Data from the present study showed very few growth differences following flutamide therapy in synostotic rabbits. Although there were differences in bodyweight after 10 days of age (the experimental group exhibited greater weight), this was seen before any treatment occurred and suggests that these differences were due to normal variation in the colony. More importantly, there were no significant differences in the somatic growth control variable, metacarpal length, at any time point by group, which suggests a similar bony growth trend.

There were not many differences by groups in the cephalometric variables suggesting a limited effect of the localized flutamide treatment. A clear trend toward greater coronal suture marker separation for the experimental flutamide group compared to controls was observed. Here the ethanol controls appear to have a similar trend by 84 days as the surgical controls. At

the 42 day time point (17 days post operation), the flutamide experimental group exhibited greater growth across the coronal suture than the surgical or collagen vehicle controls, suggesting an effect on growth during this time period. However, coronal suture growth in the ethanol control group was not significantly different from the experimental group. These results indicated that increased coronal suture growth might be due to the ethanol used to reconstitute the flutamide. Indeed, given the cellular evidence intrinsic to this dissertation, ethanol treatment appeared to decrease proliferation and differentiation of osteoblasts. Literature also supports this finding, suggesting ethanol exposure causes apoptosis (Klein, 1997; Klein et al., 1996; Vignesh et al., 2006).

The purpose of the rescue therapy was to delay the ultimate fusion of the coronal suture in this model, and facilitate craniofacial growth, specifically in the antero-posterior dimension. These animals tended to have the propensity for severe brachycrania. Thus, although the flutamide therapy may have been mildly successful in the maintenance of growth across the coronal sutures, significantly different cephalometrics measures for craniofacial length, cranial base length, cranial length, cranial width, cranial height, cranial index and even intracranial volume, would be important to establish that this therapy produced the intended effect.

If the flutamide therapy contributed to correction of growth, the expectation would be for a greater craniofacial length in the experimental group. These data did not provide evidence for any real variation among the groups for this measure. In addition, the analysis of variance revealed no differences between groups at any time point. Consequently, the flutamide therapy was not deemed useful for completely rescuing coronal sutures destined to undergo fusion and subsequent correction of craniofacial growth.

A successful therapy would be expected to induce a greater cranial length. Results suggest that the ethanol controls may have a greater growth pattern than the other groups. Only at the 25-day time point does it appear that the experimental group has the greatest mean cranial length. Again, here the analysis of variance revealed no differences by group by age. The cranial base length results are equally as poor, suggesting that the collagen vehicle with protein controls to have the greatest mean base length at all time points. In addition, it appears as though the surgical controls have the greatest change in growth between 42 and 84 days. The analysis of variance demonstrated the same relationship.

For both cranial width and cranial height, a decrease in these mean values would be expected for a successful craniofacial surgical intervention. The experimental group appears to have larger values for cranial with by 84 days than the controls. Cranial height on the other hand, demonstrated a possible decrease in the growth curve for the experimental group. Nevertheless, there were no statistically significant differences by group or by age after surgical intervention for cranial width or cranial height.

It has been suggested that craniosynostosis can alter intracranial volume (Cohen and MacLean, 2000; Mooney et al., 1998a; Mooney et al., 2007b). With his in mind, it follows that a successful surgical cytokine intervention would lead to differences in intracranial volume. Measuring length by width by height has been established as an appropriate relative approximation for intracranial volume (Cray et al., 2008; Morris et al., 2009; Rogers, 1980). According to these measures, it appears that the collagen vehicle group exhibited the greatest cranial volume by 84 days. There were no statistically significant differences in intracranial volume at 42 or 84 days, for group by age.

These results seem to suggest that even though the experimental group enjoyed minimally more growth across the coronal suture, craniofacial growth and intracranial volumes were not improved. It will be important to statistically compare these results to other molecular therapies used in this model. Possible reasons for the lack of an overarching effect may be due to problems with the surgical vehicle. As stated above the flutamide was viscous and neither phosphate buffered saline nor was water able to reconstitute the hormone receptor blocker. Thus, ethanol was utilized. In essence, it is possible that the resulting effect on the collagen would be to fix the vehicle after mixing. Another possible culprit for the lack luster effects of this study could be dose. As no other previous research had been implemented to study the effect of hormone receptor blockers in bone biology, or craniofacial biology more specifically, it was a shot in the dark. The cellular studies though informative are a difficult template for the abstraction of an appropriate dose. As it appears there was a biphasic response to flutamide administration in the cellular studies, our dosage implemented in vivo could have been too great or too little. In addition, the androgens might not have been overexpressed, or might not have been critical to growth in these tissues, which would render the experimental intervention ineffective. In addition, the effect that androgens have on TGFβ may not be necessary for proper TGFβ functionality. This functionality could have been maintained regardless of the experimental intervention by the presence of another hormone, or protein interaction. A final explanation for the results of this study would be that there is no positive indication, that androgen receptors are located in the osteogenic fronts or in the sutural ligament of the coronal suture, as suggested by Lin et al. (2004),. Again, this lack of localized androgen receptor would have rendered the experimental therapy ineffective.

4.3.2 Comparison to Previous Studies

Published research on the craniosynostotic model has focused on post-operative resynostosis after coronal suturectomy. A notable exception is Chong et al. (2003). In this experiment the same methodology was implemented as here. However, TGFβ3, thought to inhibit synostosis, was used as a therapy. Chong et al. demonstrated that a high dose of TGFβ3 rescued fused sutures and allowed for greater growth across the coronal suture, about a 4.5 mm increase by day 84. Comparing the data gathered in this present study to that of Chong et al., (2003); high dose TGFB3 appeared to have a greater effect on the coronal suture growth than flutamide therapy. The flutamide group mean coronal marker distance was 4 mm. The crux of the Chong et al. (2003) study was histomorphometry, which demonstrated again greater width at the suture compared to control animals. Thus, TGFB3 therapy was identified as a method for inhibiting coronal synostosis.

The unoperated delayed onset craniosynostotic rabbit model, craniometrics have been reported (Burrows et al., 1995). Average cranial vault width at 84 days of age was reported to be 32 mm (+/-) .226 mm. The animals in the present study all displayed less cranial width than these unoperated animals. The coronal marker separation for the present study did not appear to differ from that reported by Burrows et al., both being about 4mm. These comparisons again indicated that this surgical intervention had very limited usefulness. Similar to what was found in Chong et al. (2003), Burrows et al. (1995) reported slightly larger values for cranial height than those reported in this present study. However, it appears that cranial length in the flutamide study was similar to the length found for the unoperated data in Burrows et al. (1995), suggesting any differences in cephalometric data in the flutamide study may be due to inherent growth variation in the model.

Early onset craniosynostotic occurs in the craniosynostotic rabbit colony (Mooney et al., 1994a; Mooney et al., 1994b) when the coronal suture completely fuses at or by 10 days after birth. This model has also been utilized for attempts at intervention in the post-operative resynostosis after coronal suturectomy surgery. Noggin, a BMP inhibitor (Cooper et al., 2007) and an antibody to decrease TGF-B2 (Mooney et al., 2007a; Mooney et al., 2007b), have been shown to inhibit osteoblast activity and delay reossification of the suture area. Noggin, BMP, and TGF-β2 likely effect the osteogenic fronts and the underlying dura. Lin et al. (2004), identified androgen receptors in the underlying dura of the coronal suture in a mouse model. Thus, it may be a worthwhile to target the underlying dura in the early onset craniosynostotic with flutamide to inhibit a potential osteogenic signal.

4.4 IMPLICATIONS FOR CRANIOFACIAL BIOLOGY

Androgens are important for their established effect on bone maintenance, as well as the effect on bone development. Variations in serum testosterone level may affect cranial shape through cell and molecular factors and pathways in bone cell biology or directly through mediation of muscular development. Since serum testosterone levels vary between individuals in a continuous fashion (Andersson et al., 2003; Brambilla et al., 2007), a similar range may also exist for its effect on craniofacial growth. Cranial shape may be influenced by androgen effects on the suture. The adrenal androgens can work though androgen receptors and other pathways. Thus, the presence of the receptor is not necessary for an androgenic effect. It appears from the data in the present study that testosterone can increase the amount of differentiation produced by BMP. This result suggests that certain levels of testosterone can cause an increase in osteoblast

activity at the suture and other cranial derived bone. Testosterone may also affect other pathways such as the TGFβ or FGF pathways. The present study showed a biphasic dose effect on both osteoblast proliferation and differentiation. Thus, there is not a simple relationship indicating that more testosterone leads to more differentiation.

Androgenic exposures have been shown to result in larger calvarial dimensions, anteroposterior jaw discrepancies, increase in skull length, and inhibition of calvarial growth (Barrett and Harris, 1993; Noda et al., 1994; Fujita et al., 2004). These studies highlight a genetic-epigenetic-environment interaction. Androgens are intrinsic to the endocrine system via the established pathway, but can also be absorbed from exposure via the environment.

The human syndrome, Antley Bixler, is also indicative of an androgen effect on suture biology and growth. Severe cases of Antley Bixler result in trapezoidocephaly, while less severe cases result in coronal suture synostosis. Research also suggests that it may be possible that Antley Bixler lies toward the severely affected end of the congenital adrenal hyperplasia spectrum. Antley Bixler can be caused by maternal virilization, the overexpression of androgen. There is seemingly contradictory evidence to virilization and expression of excess androgen. Female patients diagnosed with Antley Bixler syndrome exhibit the urogenital characteristics of early androgen exposure. Males exhibit the opposite effect, an undervirilization. The expression of excess androgen by these patients postnatally is not normally found. The hallmark hormonal overexpression is of pregnenolone and progesterone, which are precursors along the glucocorticoid and adrenal pathways. These hormones are located well upstream of the sex steroid target cells. In a small number of cases, it was shown that both the father and mother of Antley-Bixler patients had slightly elevated levels of pregnenolone and progesterone, suggesting they were heterozygous carriers. In addition, the mother of Antley-Bixler patients may also

express low levels of oestriol, an estrogenic hormone commonly screened for when birth defects are suspected (Cragun and Hopkin, 2005; Roth et al., 2000; Shackleton et al., 2004; Warmann et al., 2000; Yamamoto et al., 2001).

Testosterone, an endogenous variable, can be modified by environmental variables or act as a teratogen upon exposure (Herbst, 1973; Limbird and Taylor, 1998; Molsted et al., 1997). Craniosynostosis likely has a multifactorial threshold model, similar to that described for cleft lip and palate. If a genetic predisposition for an anomaly is coupled with an environmental factor, which can exacerbate the existing predisposition, a more severe phenotype will result. A better understanding of the gene-environment interaction could aid in diagnosis and management of craniofacial anomalies (Murray, 2002).

Growth factors, such as TGFβ1, TGFβ2, and TGFβ3, have been associated with suture development in humans, rats, and rabbits. It has been noted that TGFβ1 and TGFβ2 were associated with sutural fusion. In addition, TGFβ3 plays a role in keeping sutures patent (Mooney et al., 2007a; Mooney et al., 2007b; Opperman and Ogle, 2002; Poisson et al., 2004). Therapies developed to rescue sutures from osseous have been developed using TGFβ cytokine therapy in a slow-release collagen gel. These therapies have had some success preventing further synostosis in both rabbit (Chong et al., 2003) and rat (Opperman et al., 2002) sutures. The present study described an attempt to rescue a suture destined to undergo fusion, via an androgen receptor blocker. Given the cellular data, it seemed to follow that flutamide administration might reduce osteoblast differentiation. Further, the cell data indicated flutamide administration might prolong sutural growth at the treated suture. In addition, androgens had a known effect on TGFβ2, which the craniosynostotic rabbit model overexpresses. Although, greater growth was observed across the coronal suture in flutamide treated animals in the present study, this therapy

was not successful in correcting craniofacial growth. In an androgen-compromised model, a positive result may have been observed. The present study suggests flutamide administration was insufficient to modify overall suture biology. Thus, although in vitro, sutural, and calvarial derived osteoblast cells were modified to increase differentiation upon administration of testosterone co-cultured with BMP, and this effect was successfully blocked by flutamide administration, this was not successfully modeled in vivo.

4.5 LIMITATIONS AND FUTURE DIRECTIONS

Future directions concerning cellular studies include additional quantification of cellular morphologies, specifically calcification, via standard alizarin red stains or challenge with osteogenic media co-cultures with testosterones for quantification of proliferation and differentiation. In addition, many permutations upon the known bone pathway could be addressed, including co-cultures with the fibroblast growth factors or the TGFβ isoforms, which are of particular interest to suture biology. In addition, multiple possibilities exist for better quantification of the hormone pathway involvement in normal osteoblast biology as well as synostotic suture cells of osteoblast lineage. These possibilities include challenging cells with more potent hormone, dihydrotestosterone downstream of the 5α reductase enzyme, or those adrenal androgens upstream of the conversion to the estrogenic pathway, including androstendione and dehydroepiandosterone.

There were severe limitations for androgen receptor identification in the rabbit model due to the lack of a commercially available antibody suitable for use. For the continued study of androgen receptor in the affected rabbit model, it will become necessary to identify sufficient

funding to allow a custom-made antibody to be purchased. Once this becomes reality, these studies will be repeated to correctly determine presence or absence of androgen receptors not only in the cells and tissue of the osteogenic front, but also the underlying dura mater of the synostotic animal model.

There were also several limitations to the in vivo growth studies. The bone pathway logic was sound in the sense that flutamide administration should decrease androgen receptor activity at the sutural fronts, perhaps resulting in a decrease in TGFβ2 expression. However, no published research has applied this treatment clinically to craniofacial growth, which did not allow for a proper decision tree for dosage or method of administration. It is possible, given the cellular data intrinsic to this dissertation, that the biphasic responses of flutamide administration, a higher or lower dose may have been more effective. Another problem was the vehicle, which was extremely viscous upon administration due to the necessity to reconstitute the flutamide from a solid. For this issue another route of administration or another androgen receptor blocker, cyproterone acetate (Broulik et al., 1976; Kauli et al., 1997; Mulder et al., 1987) may prove more effective. To reiterate, Lin et al. identified androgen receptor presence in late gestation fetal mice. Particularly, they found receptor in the posterior frontal suture dura, posterior frontal osteogenic front, coronal suture dura, sagittal suture dura, and the sagittal suture osteogenic front. Although the craniosynostotic rabbit model is the best model to study potential therapies for the rescue of sutures destined to undergo resynostosis, future research should first replicate both suture rescue and postoperative resynostosis after surgical excision. This can be accomplished by replicating published methodologies in a mouse or other rodent (Cooper et al., 2009; Opperman et al., 2002), or by building on the Lin et al work which has positively identified androgen receptors at the osteogenic fronts and the underlying dura associated with

this suture. Finally, as Lin et al identified androgen receptors in the underlying dura of the coronal suture in the mouse, a suturectomy study, in the craniosynostotic rabbit model, utilizing an androgen receptor blocker as a therapy, specifically targeting the underlying dura, would be a logical step. Other hormone research may also become important for their specific effects on suture biology, including estrogens, and the thyroid hormones. A stronger possibility for an effect by relaxin administration, which has a specific relationship with collagen organization, which is important to within suture organization and eventual sutural fusion also, may prove to be interesting.

APPENDIX A

WITHIN SUBJECT ANOVAS

A 6 x 2 x 2 mixed ANOVA was performed on cellular proliferation response normalized to baseline proliferation response as a function of testosterone dose, phenotype and bone type. The within subject independent variable was dose with 6 levels (-12,-14,-16,-20,-24, and -30 mols. testosterone). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone). The assumption of normality was violated for synostotic suture bone at -12, -20, -24,-30), $p=.001$. An inverse function allowed for normality for all permutations. Mauchly's test of sphericity was violated $p<.001$. Thus a Greenhouse Geiser correction was implemented. The independence of subjects assumption was met. There were no significant differences in cellular proliferation as a function of dose by phenotype and bone type, dose by phenotype, dose by bone type, phenotype by bone type, dose, phenotype, or bone type, $p>.05$, for within subject or between subject effects.

A 6 x 2 x 2 mixed ANOVA was performed on cellular proliferation response normalized to baseline BMP proliferation response as a function BMP co-cultured with testosterone dose, phenotype and bone type. The within subject independent variable was dose with 6 levels (-12,-14,-16,-20,-24, and -30 mols. testosterone). The between subject variables were phenotype (wild

versus synostotic) and bone type (suture versus non suture bone). The assumption of normality was violated for synostotic suture bone treated with BMP and all doses of testosterone, as well as wild type suture bone treated with -14 mols. A natural log transformation allowed for normality for all permutation with the exception of synostotic suture bone at -14 mols (W=.750, p=.013). ANOVA should be robust against this violation. Mauchly's test of sphericity was violated p<.001. Thus a Greenhouse Geiser correction was implemented. The independence of subjects assumption was met. There were no significant differences in cellular proliferation as a function of dose by phenotype and bone type, dose by phenotype, dose by bone type, phenotype by bone type, phenotype, or bone type, p>.05, for within subject or between subject effects. There was a significant within subject difference by dose, F=3.909, df 2.146, 27.895, p=.029. Post hoc least squared differences. Testosterone -14 (χ=.2016, SD .4435) demonstrated significantly greater proliferation response than testosterone -12 dose (χ=.0952, SD .4423), p=.04, testosterone -20 dose (χ=-.0124, SD .3285), p=.04, and testosterone -24 dose (χ=.0195, SD .3468), p=.016. Testosterone -16 dose (χ=.2130, SD .4782) was significantly greater than testosterone -20, p=.03, and -24, p=.013, doses.

A 6 x 2 x 2 mixed ANOVA was performed on cellular differentiation response normalized to baseline differentiation response as a function of testosterone dose, phenotype and bone type. The within subject independent variable was dose with 6 levels (-12,-14,-16,-20,-24, and -30 mols. testosterone). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone). The assumption of normality was violated for synostotic suture bone at -12, -14, -24,-30), in addition to synostotic non suture, and wild type suture bone at -30 dose, p=.001. A natural log function allowed for normality for all permutations, except synostotic suture bone at -24 dose (W=.682, p=.002, and T-30 (W=.704,

p=.004). ANOVA should be robust against these violations. Mauchly's test of sphericity was violated p=.031. Thus a Greenhouse Geiser correction was implemented. The independence of subjects assumption was met. There were no significant differences in cellular differentiation as a function of dose by phenotype and bone type, dose by phenotype, dose by bone type, phenotype by bone type, dose, phenotype, or bone type, p>.05, for within subject or between subject effects.

A 6 x 2 x 2 mixed ANOVA was performed on cellular proliferation response normalized to baseline BMP differentiation response as a function BMP co-cultured with testosterone dose, phenotype and bone type. The within subject independent variable was dose with 6 levels (-12,-14,-16,-20,-24, and -30 mols. testosterone). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone). There were multiple violations of normality. An inverse transformation allowed for normality for all permutation. Mauchly's test of sphericity was violated p<.001. Thus a Greenhouse Geiser correction was implemented. The independence of subjects assumption was met. There were no significant differences in cellular differentiation as a function of dose by phenotype and bone type, dose by phenotype, dose by bone type, phenotype by bone type, dose, phenotype, or bone type, p>.05, for within subject or between subject effects.

A 2 x 2 x 2 mixed ANOVA was performed on cellular proliferation response normalized to baseline proliferation response as a function of flutamide dose, phenotype and bone type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone).The assumption of normality was met for all permutations. The assumption of homogeneity of

variance and homogeneity of covariance was met for each analysis Box's M = 6.683, F (6, 661.59) = .625, p= .710; Mauchly's W = 1.000; Box's M = 5.265, F (6, 661.59) = .492, p= .814; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. There were no significant differences in cellular proliferation as a function of flutamide dose by phenotype and bone type, dose by phenotype, dose by bone type, phenotype by bone type, dose, phenotype, or bone type, p>.05, for within subject or between subject effect.

A 2 x 2 x 2 mixed ANOVA was performed on cellular proliferation response normalized to baseline BMP proliferation response as a function of BMP co-cultured with flutamide dose, phenotype and bone type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone).The assumption of normality was met for all permutations. The assumption of homogeneity of variance and homogeneity of covariance was met for each analysis Box's M = 7.7238, F (6, 661.59) = .677, p= .668; Mauchly's W = 1.000; Box's M = 10.264, F (6, 661.59) = .960, p= .452; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. Dose was significant for both comparisons with F -6 BMP co-culture treatment (χ=.9805, SD .0647) exhibiting greater proliferation than F-8 BMP co-culture treatment (χ=.9255, SD .0684), F=8.988, df 1,8, p=.017; and F -8 BMP co-culture treatment (χ=.9851, SD .1465) exhibiting greater proliferation than F-10 BMP co-culture treatment (χ=.8586, SD .1376), F=10.264, df 1,8, p=.008. In addition, the only other significant difference was a three way interaction for the comparison for -6 and -8 dose owing to the F -8 synostotic non suture bone exhibiting a lower than expected mean value (χ=.8843, SD .0334).

A 2 x 2 x 2 mixed ANOVA was performed on cellular proliferation activity on cells treated with testosterone and flutamide to block activity by dose, phenotype and bone type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone). The assumption of normality was met for all permutations. The assumption of homogeneity of variance and homogeneity of covariance was met for each analysis Box's M = 5.613, F (6, 661.59) = .525, p= .790; Mauchly's W = 1.000; Box's M = 11.164, F (6, 661.59) = 1.044, p= .395; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. The only significant difference was for the interaction dose by phenotype , F=5.844, df 1,8, p=.042, due to the synostotic mean block response for -8 mol (χ=.056, SE .043) being greater than that for -10 mol (χ= -.047, SE .087), and the inverse relationship existing for the wild type phenotype, -10 exhibiting a greater mean decrease (χ=.107, SE .073) than -8 mol treatment (χ=.022, SE .036).

A 2 x 2 x 2 mixed ANOVA was performed on cellular proliferation activity on cells treated with BMP, testosterone and flutamide to block activity by dose, phenotype and bone type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone). The assumption of normality was met for all permutations. The assumption of homogeneity of variance and homogeneity of covariance was met for each analysis Box's M = 6.122, F (6, 661.59) = .572, p= .752; Mauchly's W = 1.000; Box's M = 14.808, F (6, 661.59) = 1.385, p= .218; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. The

only significant difference was for dose, flutamide -6 mol (χ=.0793, SD .0645) blocking significantly more activity than flutamide -8 mol (χ= -.0087, SD .0635), F=16.162, df 1, 8, p=.004.

A 2 x 2 x 2 mixed ANOVA was performed on cellular differentiation response normalized to baseline proliferation response as a function of flutamide dose, phenotype and bone type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone).The assumption of normality was met for all permutations. The assumption of homogeneity of variance and homogeneity of covariance was met for each analysis Box's M = 631.376, F (9, 733.426) = 1.870, p= .06; Mauchly's W = 1.000; Box's M = 5.958, F (6, 661.59) = .557, p= .765; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. There were no significant differences in cellular differentiation as a function of flutamide dose by phenotype and bone type, dose by phenotype, dose by bone type, phenotype by bone type, dose, phenotype, or bone type, p>.05, for within subject or between subject effect.

A 2 x 2 x 2 mixed ANOVA was performed on cellular differentiation response normalized to baseline BMP proliferation response as a function of BMP co-cultured with flutamide dose, phenotype and bone type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone).The assumption of normality was met for all permutations, with the exception of wild type suture bone, W=.753, p=.01. ANOVA should be robust against this violation. The assumption of homogeneity of variance and homogeneity of covariance was met

for each analysis Box's M = 17.751, F (9, 733.428) = 1.058, p= .392; Mauchly's W = 1.000; Box's M = 2.257, F (6, 661.59) = .221, p= .973; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. Dose was significant for comparison of F -6 BMP co-culture treatment (χ=1.0772, SD .2795) exhibiting greater differentiation than F-8 BMP co-culture treatment (χ=.9331, SD .1784), F=10.761, df 1, 8, p=.011. There were no other significant differences.

A 2 x 2 x 2 mixed ANOVA was performed on cellular differentiation activity on cells treated with testosterone and flutamide to block activity by dose, phenotype and bone type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone). The assumption of normality was met for all permutations. The assumption of homogeneity of variance and homogeneity of covariance was met for each analysis Box's M = 16.740, F (9, 733.428) = .998, p= .440; Mauchly's W = 1.000; Box's M = 3.374, F (6, 661.59) = .316, p= .929; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. The interaction dose by phenotype was significant, F=12.179, df 1, 8, p=.008, due to the synostotic mean block response for -10 mol (χ=.064, SE .071) being greater than that for -10 mol (χ= .014, SE .084), and the inverse relationship existing for the wild type phenotype, -8 exhibiting a greater mean decrease (χ=.334, SE .073) than -8 mol treatment (χ=.192, SE .060). The 3 way interaction was also significant, F=26.560, df 1, 8, p=.001, which appeared to be due to a very small mean block (χ= -.055, SE .090) for synostotic non suture bone at -10 mol.

A 2 x 2 x 2 mixed ANOVA was performed on cellular differentiation activity on cells treated with BMP, testosterone and flutamide to block activity by dose, phenotype and bone

type. Two separate analyses were conducting comparing the within subject independent variable flutamide dose with 2 levels (-6 and -8 mols; -8 mols and -10 mols). The between subject variables were phenotype (wild versus synostotic) and bone type (suture versus non suture bone). The assumption of normality was met for all permutations. The assumption of homogeneity of variance and homogeneity of covariance was met for each analysis Box's M = 19.880, F (9, 733.458) = 1.185, p= .301; Mauchly's W = 1.000; Box's M = 11.459, F (6, 661.59) = 1.071, p= .378; Mauchly's W = 1.000, respectively. Independence of subjects assumption was met. The only significant difference was for dose, flutamide -8 mol (χ=.1865, SD .1995) blocking significantly more activity than flutamide -6 mol (χ= .0608, SD .2101), F=16.694, df 1, 8, p=.004.

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