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Osteoblast differentiation profiles define sex specific gene expression patterns in craniosynostosis

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Abstract

Single suture craniosynostosis (SSC) is the premature fusion of one calvarial suture and occurs in 1-1,700-2,500 live births. Congenital fusion of either the sagittal, metopic, or coronal sutures represents 95% of all cases of SSC. Sagittal and metopic synostosis have a male preponderance (3:1) while premature fusion of the coronal suture has a female preponderance (2:1). Although environmental and genetic factors contribute to SSC, the etiology of the majority of SSC cases remains unclear. In this study, 227 primary calvarial osteoblast cell lines from patients with coronal, metopic, or sagittal synostosis and unaffected controls were established and assayed for ALP activity and BrdU incorporation (n=226) as respective measures of early stage osteoblast differentiation and proliferation. Primary osteoblast cell lines from individuals with sagittal synostosis demonstrated higher levels of ALP activity and reduced proliferation when compared to control lines. In order to address the sex differences in SSC types, the data was further stratified by sex. Osteoblasts from males and females with sagittal synostosis as well as males with metopic synostosis demonstrated higher levels of ALP activity when compared to sex matched controls, and males with sagittal or metopic synostosis demonstrated reduced levels of proliferation. In order to elucidate genes and pathways involved in these observed phenotypes, correlation analyses

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comparing ALP activity and proliferation to global gene expression was performed. Transcripts related to osteoblast differentiation were identified both differentially up and down regulated, correlated with ALP activity when compared to controls, and demonstrated a striking sex specific gene expression pattern. These data support that the dysregulation of osteoblast differentiation plays a role in the development of SSC and that genetic factors contribute to the observed sex related differences.

Keywords

Osteoblasts; Osteoblast differentiation; Craniosynostosis; Gene expression analysis

1. Introduction

The flat bones of the calvaria develop through intramembranous ossification that involves the direct condensation of mesenchymal cells where intramembranous ossification is initiated.[1, 2] This differs from endochondral ossification, the process through which long bones and the skull base are formed, where cartilage differentiates into bone. Sutures are the fibrous tissue or joints that are maintained between the bones of the calvaria. This intrasutural mesenchyme is believed to contain undifferentiated and proliferative osteogenic stem cells that then differentiate into osteoprogenitor cells.[2-4] Sutures allow for cranial vault expansion to accommodate the growing brain and the deformation of the skull during childbirth.[3, 5-7] The calvaria is comprised of five major bones: the paired frontal and parietal bones, and the occipital bone. The four major sutures are the sagittal, metopic, coronal, and lambdoid sutures. Normally the metopic suture fuses between 3-9 months of age [8] while the coronal, lambdoid, and sagittal sutures fuse between 22-39 years of age.[9] Premature fusion of the sutures, known as craniosynostosis, can be syndromic (associated with consistent extracranial malformations) or nonsyndromic.[10] Nonsyndromic single suture craniosynostosis (SSC) is the most common form with an incidence of approximately 1/1,700-2,500 live births.[11, 12] Sagittal, metopic, and coronal synostosis comprise 45%, 25%, and 25% of SSC, respectively [13]. Sagittal and metopic craniosynostosis have a male preponderance (3.5:1 [13, 14] and 3.3:1[15, 16], respectively) while coronal craniosynostosis has a female preponderance (2:1). [17, 18] Craniosynostosis is a heterogenous condition that is associated with environmental and genetic factors including maternal [19] and paternal age, [17] thyroid disease, [20] smoking, [21] intrauterine constraint [22] as a consequence of multiple gestation pregnancy, primiparity, and macrosomia. [23] Familial recurrence of SSC accounts for 6% of sagittal cases, [14] 10% of metopic cases, [16] and 8-10% of coronal cases; [17] however, the etiology of most cases of SSC still remains unclear. As craniosynostosis involves the premature ossification of the sutures, dysregulation of osteogenic processes have been evaluated. Several studies have identified altered states of osteoblast differentiation and proliferation in patients with syndromic [4, 24] and nonsyndromic craniosynostosis [24, 25] as well as in a mouse model of lambdoid synostosis. [26] In an effort to better identify and characterize the biologic phenotype of SSC, 227 primary calvarial osteoblast cell lines from individuals with sagittal, metopic, or coronal craniosynostosis, as well as individuals without SSC (controls) were established. These cell lines were directly compared with respect to osteoblast

differentiation and proliferation. Results were analyzed by suture type and sex in order to determine whether sex contributes to potential phenotypic differences. Metopic and sagittal craniosynostosis were associated with enhanced differentiation and reduced proliferation of calvarial osteoblasts. These results were correlated with expression array data to elucidate potential molecular and biologic mechanisms driving this state of altered differentiation and proliferation. Differentially expressed genes involved in osteoblast biology were identified through this correlation analysis. Our results reveal that enhanced osteoblast differentiation may underlie the cause of sagittal and metopic craniosynostosis and that there are distinct sex specific gene expression profiles among cases.

2. Materials and Methods

2.1 Ethics statement

This is a HIPAA compliant study. Written informed consent from all participants with single-suture craniosynostosis was obtained and waiver of consent was obtained from Seattle Children's Hospital's Institutional Review Board (IRB) for the use of anonymous control samples. Independent prospective IRB approval was obtained from each participating institution including Seattle Children's Hospital, Northwestern University in Chicago, Children's Health Care of Atlanta, and St. Louis Children's Hospital.

2.2 Participant enrollment

As previously detailed in [27], 84% of eligible individuals with craniosynostosis were enrolled in this study. CT scans confirmed the diagnosis of isolated sagittal, metopic, or unilateral coronal synostosis. Criteria for exclusion included the presence of major medical conditions such as syndromic craniosynostosis, cardiac defects, seizure disorders, cerebral palsy, and health conditions requiring surgical intervention, presence of three or more minor extra-cranial malformations; or presence of other major malformations. Control samples were obtained from patients undergoing a craniotomy for reasons other than craniosynostosis (e.g. brain tumor, hydrocephalus, etc.) or autopsies. Control samples were not obtained from individuals with disorders that affect bone such as skeletal dysplasias.

2.3 Culture of calvarial osteoblasts

For participants with craniosynostosis, calvarial bone fragments were obtained from otherwise discarded tissues during surgical reconstruction while for control samples, calvarial tissue was obtained from anonymous surgical or autopsy specimens. Calvaria fragments were transported from the site of harvest to our laboratory in Waymouth media (WM) (Sigma, St. Louis, MO) supplemented with 2X antibiotic/antimycotic solution (GE Hyclone, Logan, UT) and 10% fetal bovine serum (FBS) (GE Hyclone, Logan, UT). Tissue was rinsed in WM and surrounding soft tissue was removed. Using a sterile scalpel blade, calvaria was cut into 1-2mm pieces and 2 pieces per well were cultured in 12-well plates at 37°C, 5%CO₂, and 99% humidity. Upon confluence (approximately 3-6 weeks), cells were washed with PBS, trypsinized with 0.05% Trypsin-EDTA (GE Hyclone, Logan, UT), and passaged into T75 flasks. After confluence (1-2 weeks), cells were cryogenically stored in freezing media containing 90% FBS and 10% dimethyl sulfoxide in a liquid nitrogen freezer.

2.4 RNA extraction and microarray processing

Cell lines were thawed and cultured to confluence in T25 flasks then passaged to a density of 175,000 cells per 25cm². In all, 249 cell lines had RNA isolated of which 100 were osteoblast cell lines from individuals with sagittal synostosis (sagOBs), 49 metopic (metOBs), 50 coronal (corOBs), and 50 controls (xOBs). For this study, expression array data from samples that did not have corresponding ALP activity and BrdU incorporation data were excluded (22 osteoblast cell lines). At 75% confluence, cells were subjected to RNA extraction using Roche High Pure miRNA Isolation Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. RNA integrity was assessed using the Agilent 2100 Bioanalyzer with only samples passing quality control run on Affymetrix Human Gene 1.0 ST arrays on which 28,869 genes are represented. Raw microarray data was processed and analyzed with Bioconductor [28] and normalized with the RMA method as implemented in the Bioconductor affy package. Microarray quality control was performed as previously described. [27]

2.5 Primary osteoblast culture for characterization

Available cell lines were randomized into batches of ten samples where each batch contained four sagOBs and two each of metOBs, corOBs, and xOBs. In all, alkaline phosphatase was run on 227 cell lines which consisted of 87 sagOBs, 44 metOBs, 49 corOBs, and 47 xOBs (226 for BrdU having only 43 metOBs). The reduction in cell lines grown (from 249 to 227) was a result of either a shortage of cell lines or samples that did not grow. Osteoblast cell lines were cultured as described above. At confluence, each line was plated onto two 96-well plates at 5×10³ cells in 0.1ml of WM per 0.32cm² in sextuplicate. After overnight culture allowing for adhesion to the plate, the media was changed and alkaline phosphatase and proliferation assays were performed.

2.5.1 Alkaline phosphatase (ALP) assay—Forty eight hours after plating, the media was removed, the wells rinsed once with 1X PBS (Thermo Fisher Scientific, Waltham, MA), and 0.2ml p-nitrophenyl phosphate (pNPP) (Sigma, St. Louis, MO) was added to three wells and incubated for 1 hour at room temperature in the dark. After 1 hour, 0.05ml 3N NaOH was added to each well to stop the reaction and the sample was analyzed on a SpectraMAX 190 spectrophotometer at 405nm.

2.5.2 Bicinchonic acid (BCA) assay—A bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA) for total protein quantitation was performed in triplicate according to manufacturer's protocol and the plate was read at 562nm. Cells were lysed in 0.05ml Mammalian Protein Extraction Reagent (M PER) (Thermo Fisher Scientific, Waltham, MA). Triplicates were averaged and adjusted for blank measurements. ALP activity was defined by dividing the values from the pNPP assay by the values from BCA total protein to represent normalized alkaline phosphatase activity per unit total protein. Averaged values by case type were compared to controls and stratified by sex. Statistical analysis was performed using a two-tailed t-test with Welch's correction for unequal variances.

2.5.3 Proliferation assay—In order to assess proliferation rates, BrdU incorporation was measured using the BrdU Cell Proliferation Kit (EMD Millipore, Billerica, MA). The assay was carried out according to manufacturer's instructions. Twenty four hours after plating, 1X BrdU reagent (diluted in WM), was added to three wells and incubated for 24 hours. Plates were read at two wavelengths, 450/550nm where readings from 550nm were subtracted from 450nm as background. Triplicates were averaged and background reading subtracted. Averaged values by case type were compared to controls and stratified by sex. Statistical analysis was performed using a two-tailed t-test with Welch's correction for unequal variances.

2.6 Correlation of ALP activity and BrdU data with ALPL expression

ALP activity and BrdU incorporation data were correlated with alkaline phosphatase (*ALPL*) expression data using linear regression with the resulting Pearson product-moment correlation coefficient to measure the linear relationship between ALP activity or BrdU and *ALPL* expression (intensity units log₂ expression). Given that there is no sole gene equivalent to BrdU incorporation, a correlation analysis comparing BrdU data from all samples to the gene expression array data was performed. Genes with a correlation coefficient of 0.35 or higher (n=201) were analyzed with GeneMANIA software in order to identify the biological processes and roles most correlated with BrdU incorporation

2.7 Correlation of ALP activity with genomic expression array data

In order to identify differentially expressed genes correlated in case samples with high ALP activity and reduced proliferation, two-mixed effects models were fitted using R/Bioconductor's [28] limma package [29, 30] to calculate differential gene expression, taking into account diagnosis (sagittal, metopic, coronal craniosynostosis or control), sex, either ALP activity or BrdU incorporation, and the batch effect of date of sample processing. The limma methodology calculates a p-value for each gene using a modified t-test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. This method of detecting differentially expressed genes draws strength across genes for more robust and accurate detection of differential expression. Such an adjustment has repeatedly been shown to avoid an excess of false positives.[31] The log-fold changes between cases and controls were identified at two values of Alkaline Phosphatase activity, low, 1U (ALP1), and high, 128U (ALP7) where $U = (OD_{405}) / (BCA_{total\ protein})$. These two points represented the range of values of ALP activity. For proliferation, two values of BrdU^{Abs} (Abs= $OD_{450} - OD_{550}$), low of 0(BrdU0^{abs}) and high of 1.6 (BrdU1.6^{Abs}) represented the range of values for BrdU incorporation in the cell lines. Based on ALP activity, *ALPL* expression, and BrdU data, three groups, female and male sagOBs (sagOB-F and sagOB-M) and male metOBs (metOB-M) were selected for comparison using the correlation analysis of expression array data and high ALP activity and low BrdU incorporation. Given that there were no significant differences between xOBs and corOBs with regard to ALP activity and BrdU incorporation, corOBs were excluded from further correlation study. Transcripts with $p < 0.05$ and fold change $> |1.5|$ were included in the analysis. Correlation analyses for female and male sagOBs and male metOBs was analyzed with BioVenn[®] in order to identify shared and unique gene sets.[32]

2.8 Identification of genes related to osteoblast differentiation

In order to identify genes that play a role in osteoblast biology, gene lists from the subsets were filtered against 23 relevant Gene Ontology terms using QuickGO (<http://www.ebi.ac.uk/QuickGO/>). (Supplemental Table 1)

2.9 qRT-PCR validation

Primer sequences for CLEC3B, DKK2, EDN1, IGF1, MME, MSX2, PTGS2, RPL13A, SMOC1, and *TGFB2* were obtained from Harvard PrimerBank.[33] Primer efficiency and validation was performed prior to use. Replicability, slope, and Ct values were assessed in the determination of primer efficiency. cDNA was synthesized with the SensiFast cDNA synthesis kit (Bioline, Taunton, MA). A total of 46 samples were used for validation of the array results. Ten lines each from metOB-M and sagOB-F and M, nine lines from xOB-M, and seven lines from xOB-F were chosen by high levels of ALP activity and plated in duplicate at 1ng per reaction (5ng for *MME*) with 0.4uM primer using Bioline's SensiFast Sybr Lo-Rox. Reactions were run according to manufacturer's instructions. The housekeeping gene ribosomal protein L13a (*RPL13A*) [34] was used for normalization and analysis was performed using the Ct method [35] where fold changes for each target gene and subgroup (sagOB-F, sagOB-M, and metOB-M) were calculated by the differences between the Ct values of each of the averages of the three subgroups and the Ct values of their respective controls. These values were compared to the fold changes from our expression array analyses on the same 46 samples to determine consistency in magnitude and direction of gene expression differences. Fold changes were calculated from the log2ratio (which is the difference between log transformed RMA values of cases and controls).

3. Results

3.1 Calvarial osteoblast differentiation and proliferation

A significant 32% average increase in ALP activity in sagOBs was found ($p=0.0063$). MetOBs, while not significant demonstrated a 16% average increase and corOBs exhibited no difference when compared to xOBs (Figure 1a). Proliferation data demonstrated a significant 23% average reduction in BrdU incorporation in sagOBs ($p=0.0341$) and a 17% average reduction in metOBs (Figure 1b).

3.1.1 ALPL expression in calvarial osteoblasts—*ALPL* expression from microarray experiments from samples with ALP activity and proliferation data were examined. Differences in expression levels were represented as fold changes between each case type and controls. A significant 1.5 fold increase in sagOBs compared to controls was found ($p=0.0008$). CorOBs again demonstrated an insignificant increase when compared to controls, thus validating that *ALPL* expression levels were consistent with ALP activity (Figure 1c).

3.1.2 Correlation of ALPL expression and ALP activity—In preparation for genome wide correlation analysis, the correlation between the designated marker of differentiation (ALP activity) and the transcription of *ALPL* was investigated. Establishing a correlative

relationship provides proof of principle in the utilization of expression array data to identify candidate genes correlated with ALP activity. This was similarly applied to the BrdU incorporation data given the observation that ALP activity and BrdU incorporation were found to have an inverse relationship. A strong positive correlation (Figure 2a) between ALP activity and *ALPL* expression was identified ($r=0.69$). Given the strong correlation between ALP activity and *ALPL* expression, expression array analysis was used as a means to identify candidate genes that may drive the observation of increased differentiation. Though less pronounced, a negative correlation (Figure 2b) between *ALPL* expression and BrdU incorporation (as a proxy for proliferation), was identified. Genes found through the correlation analysis of BrdU incorporation with the array dataset were analyzed using the GeneMANIA program. A striking majority of functional associations were related to the cell cycle (Supplemental Table 2). This supported the utility and relevance of analyzing expression data as it correlated with BrdU incorporation.

3.1.3 Sex differences in ALP activity, ALPL expression and proliferation—

Given the male preponderance of sagittal and metopic synostosis and female preponderance in coronal synostosis patients, the potential of sex affecting osteoblast differentiation and proliferation potential was examined. Taken together, osteoblast cell lines from all males (cases and controls) had 10% greater normalized ALP activity than all females whereas females demonstrated 19% greater BrdU incorporation than males. However, sagOB-Fs demonstrated the highest ALP activity and *ALPL* expression of all groups. (Figure 3)

3.2 Expression array correlation analysis

In efforts to elucidate potential candidate genes and pathways contributing to the state of enhanced differentiation, correlation studies analyzing ALP activity with expression data were performed. Up and down regulated genes that were strongly correlated with high ALP activity or low BrdU incorporation and differentially expressed between subgroups (sagOB-F, sagOB-M, metOB-M and controls) were identified (Figure 4). Under conditions of high ALP activity, 156 differentially expressed genes were identified in sagOB-F, 48 in sagOB-M, and 58 in metOB-M. Twenty one genes were shared between the sagOB-M and metOB-M subgroups. Notably, only two genes, *SNORD114-6* and *IGF2BP1*, had significant differences between sagOB-F and sagOB-M. Both genes were downregulated in sagOB-F but upregulated in sagOB-M, thus there was no overlap between these groups.

Similarly, when low BrdU incorporation was correlated with gene expression, 78 sagOB-M and 66 metOB-M differentially expressed genes were noted. Of these, 25 genes were shared between these two groups. Similar to the ALP correlation analysis, when sagOB-F gene expression was correlated with low BrdU incorporation, they demonstrated a very distinct expression profile comprised of 42 unique genes with no overlap between sagOB-M or metOB-M (Figure 4b). The genes identified in these subsets as differentially regulated at high ALP activity (Table 1) or reduced BrdU incorporation (Table 2) were identified using GO terms related to osteoblast biology.

3.3 qRT-PCR validation

qRT-PCR was run on the three subgroups to validate the findings from the correlation analyses using the expression array data (Table 3). This was done to demonstrate that the expression array data used to generate the correlation analysis was verifiable and validated. Nine target genes and one housekeeping gene were assayed. Fold changes were calculated based on comparisons between sagOB-Ms, metOB-Ms and xOB-Ms as well as sagOB-Fs and xOB-Fs. Validation was determined by assessing whether the fold changes from the samples run in the qRT-PCR reactions were consistent with the fold changes determined by expression array for these same samples. The goal of this validation was to demonstrate that the array data was accurate and not necessarily to replicate data from Tables 1 and 2 and Figure 4. Thus, there are instances where the composite array data may have shown a different change in expression than our validation (for example, *PTGS2*). Directionality and magnitude were both assessed. Overall, qRT-PCR results demonstrate consistency with the expression analysis data.

4. Discussion

Our study has identified a subset of craniosynostosis cases with evidence of increased differentiation. Correlation analyses comparing an early stage marker of osteoblast differentiation (ALP activity) and proliferation (BrdU incorporation) with global gene expression identified genes of interest that may drive this phenotype. A noteworthy aspect of the cellular phenotyping and expression array analysis is that although the cell lines are from the same samples, the experiments were undertaken at different times, three years apart, and we were able to demonstrate agreement of alkaline phosphatase levels via a strong positive correlation between ALP activity (cellular phenotyping) and *ALPL* expression (expression array) as well as a relationship between levels of BrdU incorporation and cell-cycle related genes. Remarkably, this phenotype can be observed *in vitro* which suggests a genetic component to the development of isolated craniosynostosis. Similar characteristics of elevated *ALPL* expression and decreased proliferation have been observed during normal osteoblast differentiation [36] as well as in a transgenic mouse overexpressing *Nell-1* [37], a gene whose expression was found upregulated in prematurely fusing and fused coronal sutures.[38] The results of the present study are also consistent with histomorphometric analysis and cell culture of calvaria from patients with nonsyndromic craniosynostosis that demonstrate increased bone formation and ALP activity in fused versus patent sutures.[25] A similar study identified an upregulation of Lim mineralization protein (*LMP*) in fused compared to patent suture cells raising the osteogenic potential at these sites.[39] More recently, it was determined that *Gli3* null mice develop lambdoid craniosynostosis and exhibit increased osteoprogenitor proliferation and differentiation. [26] Together, these data along with this present study, strongly suggests that the enhanced differentiation observed in sagittal and metopic synostosis cell lines plays a role in the development of craniosynostosis.

4.1 Effects of sex on osteoblast differentiation

The incidence of sagittal and metopic synostosis is 3.3-3.5 fold greater in males than females. Conversely, coronal synostosis is twice as common in female as males. The role

that sex may play in predisposing an individual to certain forms of craniosynostosis has been of interest. One study evaluated the role of estrogens in murine suture fusion and found that estrogens promote osteoblast differentiation [40] whereas another study observed that dihydrotestosterone increased osteoblast markers and led to sagittal suture fusion [41]. The authors present and suggest the importance of both androgens and estrogen receptors in bone formation. Sex specific differences in alkaline phosphatase activity and *Alpl* expression have been identified in the calvarial osteoblasts of several mouse strains. The authors also noted that osteoblast cultures from female mice demonstrated a reduction in *Alpl* expression over time compared to their male littermates. This suggests that not only are ALP activity and *ALPL* expression lower in females, but that the potential for osteoblast differentiation is also reduced. [42] These findings prompted us to stratify our data by sex. We hypothesized that the increased incidence of craniosynostosis in males may be due in part to higher baseline levels of *ALPL* and ALP activity or increased osteogenic potential thus predisposing males to the disease. Overall, we did observe increases of ALP activity and expression in sagOBs and metOB-M, and a general trend of males having higher ALP activity. SagOB-F, however, exhibited both higher ALP activity and *ALPL* expression than their male counterparts, suggesting that females with sagittal synostosis may represent a distinct subset with respect to pathogenesis. To explain this further, we sought to determine transcriptional changes guided by cellular phenotyping through correlating these phenotypic data with our expression array data.

4.2 Correlated genes and their roles in osteoblast biology

Previous studies have identified gene expression profiles of calvarial osteoblasts in order to elucidate mechanisms of premature suture fusion.[43-46] In our approach, we examined the relationships between cell phenotype (ALP activity and proliferation) and expression array data. The identification of differences in ALP activity and BrdU incorporation in a subset of our cases compared to controls and the availability of a robust microarray dataset [27] enabled the exploration of key gene pathways and processes. Of particular interest were genes correlated with increased ALP activity (measure of differentiation) or reduced BrdU incorporation that demonstrated significant fold change differences when compared to controls. The hypothesis was that these data could inform processes of dysregulation in osteogenic differentiation and proliferation in a subset of craniosynostosis cases. While metOB-Ms and sagOB-Ms shared transcripts involved in osteogenesis and correlated with high ALP activity, sagOB-Fs had a unique expression profile. The key transcripts with roles in ossification, mineralization, and differentiation identified using GO terms included *DKK2*, *EDN1*, *IGF1*, *MSX2*, *PTGS2 (COX2)*, *MME1*, and *SMOC1* which were all upregulated in comparison to respective controls. Endothelin-1 (*EDN1*), prostaglandin-endoperoxide synthase (*COX2*), and *DKK2*, genes unique to metOB-Ms and sagOB-Ms, promote osteoblast differentiation. ET-1 (endothelin-1 peptide) plays a key role in osteoblast metastases [47] and has been reported to stimulate osteoblast proliferation and differentiation in cultured rat calvarial cells [48]. A global gene expression study on the effects of ET-1 on mouse calvarial cells and human osteoblasts demonstrated stimulation of osteoblast differentiation via induction of genes with known roles in promoting osteoblast differentiation including *COX2*. [49] *Cox2* KO mice demonstrate reduced rates of intramembranous bone formation associated with lower levels of *Chfa1 (Runx2)* and *Osterix*

expression, two key players in bone formation. [50] Furthermore, *Cox-2* disruption in murine calvarial cultures is associated with reduced ALP activity and increased proliferation. [51] Dickkopf-related protein 2 (*DKK2*) plays a role in the terminal differentiation of osteoblasts and subsequent mineralization of the matrix. *Dkk2* null mice are osteopenic and calvarial osteoblast cultures from these mice had considerable reduction in mRNA levels of osteocalcin and osteopontin. [52] These findings strongly suggest that these three gene targets play important roles in the observed state of enhanced differentiation in osteoblasts from males with either sagittal or metopic craniosynostosis. In sagOB-F, membrane metallo-endopeptidase (*MME*) and SPARC related modular calcium binding 1 (*SMOC1*) were identified as genes positively correlated with ALP activity and differentially upregulated in sagOB-F compared to osteoblasts from female controls. *MME*, also known as *CALLA* or *CD10*, is expressed in osteoblasts and promotes osteoblast differentiation. [53] Knock down of *SMOC1* in human bone marrow stem cells leads to a significant reduction in mineralization and reduced expression of markers of osteoblast differentiation. Conversely, overexpression of *SMOC1* enhances expression of osteoblast differentiation markers such as alkaline phosphatase, bone sialoprotein, osteocalcin, and osteopontin. [54] With regard to identification of significantly altered genes correlated with decreased proliferation, *SMOC1* was also identified in metOB-Ms with reduced BrdU incorporation. Similarly, *MSX2* and *IGF1* were upregulated in sagOB-Fs with low BrdU incorporation and both *MSX2* and *IGF1* have well established roles in osteoblast differentiation. [55-57] Previous studies in our laboratory identified an *IGF1* subtype within our craniosynostosis population [58] suggesting an important role of *IGF1* in the development of craniosynostosis. Our correlation analysis also identified genes that while not found in the GO search, contribute to osteoblast biology. We identified Hedgehog interacting protein (*HHIP*) to be the second most upregulated gene (3-fold upregulated) in metOB-Ms and sagOB-Ms at high ALP. In a study looking at gene expression profiles comparing calvarial bone and suture, the authors identified an upregulation of *HHIP* in bone tissue compared to the suture. [59] In agreement with one study [45] looking at the expression profiles of prematurely fused suture tissue compared to de-differentiated cells, we found *GCA* upregulated and *LRP5* downregulated in sagOB-Fs and *DKK1* upregulated in metOB-Ms at high ALP activity (Supplemental Table 3). Our gene expression correlation analysis demonstrates key genes in osteoblast differentiation that are either up or down regulated and correlated with high ALP activity. While further proteomic and functional analyses are needed to thoroughly evaluate the roles of these genes, strong evidence has been found suggesting that the dysregulation of genes related to osteoblast differentiation may contribute to the development of nonsyndromic single suture craniosynostosis.

4.3 Control samples and age related differentiation

It is important to note that the control samples in this study were on average from older subjects than the cases. In the present study reduced proliferation in the youngest (sagittal) samples and increased proliferation in the oldest (controls) samples is observed, while other studies examining normal osteoblast cell lines have found more differentiated osteoblasts in older donors and more proliferative osteoblasts in younger donors. [60, 61] Case osteoblast cell lines, albeit younger than controls, have overall higher ALP activity suggesting increased early stage osteoblast differentiation. Furthermore, an opposite trend is noted

where higher rates of proliferation would be predicted in younger samples. This is important in that our phenotypic observations would perhaps be even more marked if the controls were more similar in age. Future studies incorporating age matched controls may strengthen our results demonstrating increased differentiation and reduced proliferation that could potentially have been muted by the fact that the control samples were older thus having baseline levels of a more differentiated, less proliferative, osteoblast phenotype.

5. Conclusion

In this study, we have identified enhanced osteoblast differentiation in cell lines derived from males and females with sagittal craniosynostosis and males with metopic craniosynostosis and reduced proliferation in males with sagittal and metopic craniosynostosis. Steps have been taken to explore the genetic control of these phenotypic observations by correlating differentiation (ALP activity) with expression array analysis. We have identified genes with significantly altered levels of expression correlated with the disease state. Osteoblast cell lines from males with either sagittal or metopic craniosynostosis were more similar in terms differentially expressed genes when in a state of enhanced differentiation than females with sagittal craniosynostosis. These results suggest not only that altered osteoblast differentiation plays a role in the development of craniosynostosis as suggested in previous studies, but also that the pathogenesis of sagittal synostosis in females may be distinct from that of males as evidenced by our identification of a unique set of genes expressed in osteoblast cell lines derived from females with sagittal synostosis. The identification of genes whose expression is correlated with differentiation and is sex specific suggests that these transcripts may play a role in the male predominance of sagittal and metopic craniosynostosis and furthermore, that females with sagittal craniosynostosis may have different genetic risk profiles. Further exploration of these findings will be assessed with ongoing studies looking at cell-matrix interactions, cellular biomechanics, genomic expression and sequencing efforts, and pathway analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

corOB	Primary calvarial osteoblast cell lines from individuals with coronal synostosis
corOB-F	females
corOB-M	males
GO	Gene Ontology
metOB	Primary calvarial osteoblast cell lines from individuals with metopic synostosis
metOB-F	females
metOB-M	males
pNPP	p-nitrophenyl phosphate
sagOB	primary calvarial osteoblast cell lines from individuals with sagittal synostosis
sagOB-F	females
sagOB-M	males
SSC	nonsyndromic single suture craniosynostosis
WM	Waymouth medium
xOB	Primary calvarial osteoblast cell lines from control samples
xOB-F	females
xOB-M	males

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Highlights

- Calvarial osteoblasts from a subset of craniosynostosis patients demonstrate increased differentiation *in vitro*.
- Correlation of cell phenotype with microarray data identifies genes of interest involved with osteoblast differentiation
- Unique gene expression profile correlated with osteoblast differentiation identified in females with sagittal synostosis

Figure 1.

Scatterplots of ALP activity (A) ($OD_{405}/(BCA_{\text{total protein}})$), BrdU incorporation (B) ($OD_{450}-OD_{550}$), and *ALPL* (C) ($\log_2$ gene expression). Each diamond on scatterplot represents an individual cell line. Significant differences were identified between sagOB and xOB with ALP activity (increased in sagOB, $p=0.0063^{**}$), BrdU incorporation (decreased in sagOB, $p=0.0341^{*}$), and *ALPL* expression (increased in sagOB, $p=0.0008^{***}$).

Figure 2.

ALPL gene expression from all samples (n=227) plotted against respective log2 ALP activity (A). *ALPL* gene expression from all samples (n=227) plotted against respective BrdU incorporation (n=226) (B). Correlation of *ALPL* expression with ALP activity and BrdU incorporation identifies a strong positive association between *ALPL* and ALP activity in all samples (A) and negative association between *ALPL* expression and BrdU incorporation (B).

Figure 3.

Scatterplots of ALP activity (A) ($OD_{405}/(BCA_{total\ protein})$), BrdU incorporation (B) ($OD_{450}-OD_{550}$), and *ALPL* (C) ($\log_2$ gene expression) separated by sex. Each diamond on scatterplot represents an individual cell line. Stratification of results by sex identifies significant differences between sagOB-F and xOB-F for ALP activity ($p=0.0008^{***}$)(A), sagOB-M and xOB-M for BrdU incorporation ($p=0.0301^*$)(B), and metOB-M, sagOB-M and xOB-M for *ALPL* expression ($p=0.0131^*$ and 0.0056^{**} , respectively)(C).

Figure 4.

Genes correlated with ALP activity and differentially expressed ($p < 0.05$, fold change $> |1.5|$) when compared to respective controls at high ALP activity (A) and low BrdU incorporation (B). Area proportional Venn diagrams demonstrate that sagOB-M and metOB-M share genes (identified in correlation analysis) in common while sagOB-F have a unique expression profile correlated with differentiation. The two genes shared by sagOB-F and sagOB-M (A) are *IGF2BP1* and *SNORD114-6* and both are downregulated for sagOB-F and upregulated for sagOB-M.

Table 1**High ALP activity**

sagOB-F					
Fold change	value	Gene	Gene description	Functions	
-1.88	0.049	LRP5	low density lipoprotein receptor-related protein 5	ossification and its regulation, bone development and morphogenesis	
3.12	0.025	ALPL	alkaline phosphatase, liver/bone/kidney	ossification, bone development and morphogenesis	
4.53	0.003	MME	membrane metallo-endopeptidase	osteoblast differentiation	
4.89	0.005	SMOC1	SPARC related modular calcium binding 1	regulation of ossification	
sagOB-M					
Fold change	value	Gene	Gene description	Functions	
-1.67	0.032	CLEC3B	C-type lectin domain family 3, member B	ossification, bone mineralization	
1.71	0.041	TGFB2	transforming growth factor, beta 2	regulation of ossification	
1.80	0.004	EDN1	endothelin 1	bone development and morphogenesis	
1.91	0.010	DKK2	dickkopf homolog 2 (Xenopus laevis)	terminal osteoblast differentiation	
1.93	0.037	FBN2	fibrillin 2	regulation of ossification	
1.98	0.041	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	ossification and bone mineralization	
metOB-M					
Fold change	value	Gene	Gene description	Functions	
-1.73	0.042	CLEC3B	C-type lectin domain family 3, member B	ossification, bone mineralization	
1.79	0.046	TGFB2	transforming growth factor, beta 2	regulation of ossification	
1.85	0.029	DKK2	dickkopf homolog 2 (Xenopus laevis)	terminal osteoblast differentiation	
2.27	0.000	EDN1	endothelin 1	bone development and morphogenesis	
2.74	0.007	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	ossification and bone mineralization	

Genes correlated with high ALP activity and differentially expressed ($p < 0.05$, fold change $> |1.5|$) with roles in osteoblast biology as identified through GO term searches. Fold changes are calculated between case group and respective sex matched controls.

Table 2

Low BrdU incorporation

sagOB-F				
Fold change	value	Gene	Gene description	Functions
1.66	0.012	GPM6B	glycoprotein M6B	ossification and its regulation
1.79	0.019	SFRP2	secreted frizzled-related protein 2	regulation of ossification, bone development and morphogenesis
1.94	0.014	MSX2	msh homeobox 2	ossification and its regulation, bone development and morphogenesis, cranial suture morphogenesis
2.16	0.025	TEK	TEK tyrosine kinase, endothelial	ossification, bone development and morphogenesis
2.36	0.017	ALPL	alkaline phosphatase, liver/bone/kidney	ossification, bone development and morphogenesis
3.07	0.035	IGF1	insulin-like growth factor 1 (somatomedin C)	ossification and its regulation, bone development and mineralization

sagOB-M				
Fold change	value	Gene	Gene description	Functions
1.72	0.008	FGF9	fibroblast growth factor 9 (glia-activating factor)	ossification
1.72	0.035	RSPO2	R-spondin 2 homolog (Xenopus laevis)	ossification and bone mineralization

metOB-M				
Fold change	value	Gene	Gene description	Functions
1.52	0.031	SMOC1	SPARC related modular calcium binding 1	regulation of ossification
1.74	0.008	ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	regulation of ossification

Genes correlated with low BrdU incorporation and differentially expressed ($p < 0.05$, fold change $> |1.5|$) with roles in osteoblast biology as identified through GO term searches. Fold changes are calculated between case group and respective sex matched controls.

Table 3

Fold changes

metOB-M and sagOB-M			
Gene	Case type	qRT-PCR	Expression array
<i>CLEC3B</i>	MET M	-1.49	-1.52
<i>CLEC3B</i>	SAG M	1.19	-1.15
<i>DKK2</i>	MET M	2.26	1.41
<i>DKK2</i>	SAG M	2.10	1.62
<i>EDNI</i>	MET M	2.16	1.37
<i>EDNI</i>	SAG M	2.94	1.41
<i>PTGS2</i>	MET M	1.11	1.08
<i>PTGS2</i>	SAG M	1.18	1.00
<i>TGFB2</i>	MET M	1.00	1.31
<i>TGFB2</i>	SAG M	2.04	1.85
sagOB-F			
Gene	Case type	qRT-PCR	Expression array
<i>IGF1</i>	SAG F	4.32	2.20
<i>MME</i>	SAG F	2.17	1.39
<i>MSX2</i>	SAG F	2.04	1.45
<i>SMOC1</i>	SAG F	2.29	1.93

qRT-PCR validation on n=10 representative cell lines each of metOB-Ms, sagOB-Fs and sagOB-Ms, n=9 of xOB-Ms and n=7 of xOB-Fs. Samples were run in duplicate and normalized to housekeeping gene *RPL13A*. Fold changes for qRT-PCR were calculated with the $2^{-\Delta\Delta C_t}$ method where

ΔC_t is the difference between averages of the C_t individual in a subgroup and C_t average of the respective controls. Fold changes for expression array were calculated as a log2 ratio. For the samples chosen for validation qRT-PCR demonstrates overall agreement with microarray data.