Age-related Changes in the Biomolecular Mechanisms of Clavicular Osteoblast Biology Affect Fibroblast Growth Factor-2 Signaling and Osteogenesis*

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The ability of immature animals to orchestrate successful calvarial ossification has been well described. This capacity is markedly attenuated in mature animals and humans greater than 2 years of age. Few studies have investigated biological differences between juvenile and adult osteoblasts that mediate successful osteogenesis. To identify possible mechanisms for this clinical observation, we investigated cellular and molecular differences between primary osteoblasts derived from juvenile (2-day-old) and adult (60-day-old) rat calvaria. Data demonstrated that juvenile osteoblasts contain a subpopulation of less differentiated cells as observed by spindle-like morphology and decreased osteocalcin production. Juvenile, compared with adult, osteoblasts showed increased proliferation and adhesion. Furthermore, following rhFGF-2 stimulation juvenile osteoblasts increased expression of collagen Iα1 (5-fold), osteopontin (13-fold), and osteocalcin (16-fold), compared with relatively unchanged adult osteoblasts. Additionally, juvenile osteoblasts organized and produced more matrix proteins and formed 41-fold more bone nodules. Alternatively, adult osteoblasts produced more FGF-2 and preferentially translated the high molecular weight (22 kDa) form. Although adult osteoblasts transcribed more FGF-R1 and juvenile osteoblasts transcribed more FGF-R2 at baseline levels, juvenile osteoblasts translated more FGF-R1 and -R2 and showed increased phosphorylation. Collectively, these findings begin to explain why juvenile, but not adult, osteoblasts successfully heal calvarial defects.

Successful calvarial ossification is a characteristic generally restricted to juvenile animals and infants younger than 2 years of age (1, 2). Since this osteogenic capacity rapidly diminishes, older children and adults with non-healing cranial defects present a difficult reconstructive challenge and a substantial biomedcal burden. Studies have documented a paracrine interaction between the underlying juvenile dura mater and the overlying cranial osteoblasts during the bone induction processes of calvarial morphogenesis, cranial suture fusion, and calvarial ossification (3–10). Previous studies have focused on the cellular and molecular differences between juvenile and adult dura mater, which are critical to calvarial bone induction. In contrast, few studies have investigated the effects of age on the cellular and molecular differences between juvenile and adult-derived calvarial osteoblasts (11).

Developmentally, mesenchymal cells enter the osteoblastic lineage and undergo a spatial and temporal progression (12, 13). Early pre-osteoblasts proliferate before maturing into bone-producing osteoblasts. Osteoblasts direct osteogenesis through the production of extracellular matrix, mineralization of matrix, and regulation of bone remodeling through resorption and deposition. For osteoblasts to proliferate, migrate, differentiate, and eventually form bone, they must be able to produce and organize a variety of cytoskeletal and extracellular matrix molecules. As osteoblasts adhere to a new environment, they initially attach to fibronectin and the cell body begins to flatten (14). At this point, the cell organizes its actin bundles and cytoskeletal proteins including integrins. These molecules then signal the cell to enter either a proliferative or differentiated state.

Progression of osteoblast differentiation corresponds to sequential gene expression. Proliferative pre-osteoblasts express early markers of differentiation including runt-related transcription factor 2 (Runx2) and collagen Iα1 (15, 16). As osteoblasts mature, they begin to express intermediate markers of differentiation including osteopontin, an extracellular matrix protein associated with matrix formation and maturation (17). Mature osteoblasts express the extracellular matrix protein osteocalcin, which is associated with increased matrix mineralization and decreased proliferation (18). The progression of osteoblast differentiation and the expression of the previously mentioned genes are tightly regulated by several factors including fibroblast growth factor-2 (FGF-2) (19).

FGF-2 has a two-pronged effect as it is an osteoblast mitogen and it stimulates bone formation as demonstrated in several mammalian models (12, 20–24). Furthermore the immature, but not mature, dura mater is a potent source of FGF-2, stimulating juvenile osteoblasts during cranial development, suture fusion, and calvarial healing (25–30). Interestingly, FGF-2 has differential effects on osteoblast biology depending on the stage of cellular maturation (12, 20–24). FGF-2 exerts its biological effect upon binding to its receptors (FGF-R 1–4) for differential regulation of osteogenesis (31). These tyrosine kinase receptors become phosphorylated upon FGF-2 binding and activate ERK and p38 MAPK signaling pathways (32). While FGF-R3 and -R4 are restricted to areas of growth plate development and cranial musculature respectively, FGF-R1

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† The abbreviations used are: FGF, fibroblast growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DAPI, 4’,6-diamidino-2-phenylindole.

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and -R2 are found in areas of the frontal and parietal bones and coronal sutures (23, 33). FGF-R1 transcription is associated with highly differentiated osteoblasts, while FGF-R2 transcription is associated with more proliferative osteoblasts (23). FGF-2 binds with equal affinity to FGF-R1 and -R2 (31, 34), thus regulation of signaling must occur not only with ligand availability, but also with receptor abundance and availability.

To investigate the effects of age on osteoblast biology and assess FGF-2 signaling in this system, we established primary juvenile (2-day-old) and adult (60-day-old) rat calvarial osteoblast cultures. The data demonstrated that juvenile osteoblast cultures, as compared with adult osteoblast cultures, contained a larger subpopulation of less differentiated immature cells, as indicated by spindle-like morphology and decreased osteocalcin production. Additionally, juvenile osteoblasts showed increased proliferation, attachment, and expression of osteogenic genes in response to rhFGF-2. Juvenile osteoblasts increased production and organization of several key cytoskeletal and extracellular matrix proteins as well as increased bone nodule formation 41-fold. Finally, juvenile osteoblasts produced less FGF-2 protein, while demonstrating increased FGF-R1 and -R2 mRNA, protein, and phosphorylation upon stimulation. These results suggest that FGF-2 caused juvenile osteoblasts to initially increase proliferation and later to become more differentiated, while it had little effect on adult osteoblasts. Taken together, the data begin to demonstrate the competency of juvenile, but not adult, animals to respond to FGF-2 stimulation and successfully orchestrate calvarial osteogenesis.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—All experiments were performed in accordance with Stanford University Animal Care and Use Committee guidelines. Sprague-Dawley rats were purchased from Charles River Laboratories Inc. (Wilmington, MA). For all animals, the first day of life was considered the first day after birth. Animals were housed in light and temperature controlled facilities and given food and water ad libitum. Osteoblasts were harvested from juvenile (2-day-old) and adult (60-day-old) rats (29). In brief, calvariae were meticulously stripped free from dura mater and overlying pericranium, washed with serial dilutions of betadine in phosphate-buffered saline, then digested 5 times for 15 min in 0.2% dispase II and 0.1% collagenase A at 37 °C (digestions 3–5 were pooled for osteoblast cultures). Cells were centrifuged for 5 min at 1000 × g and resuspended in Dulbecco's modified Eagle's medium (1×EMEM). Cultures were maintained in DMEM with 10% FBS (Gemini Bio-Products, Woodland, CA), 100 IU/ml penicillin, 100 IU/ml streptomycin, and 0.1% Fungizone (all from Invitrogen, Carlsbad, CA) at 37 °C in an atmosphere of 5% CO2. Cells were passed by trypsinization, plated in tissue culture dishes, and allowed to grow to subconfluence. Freshly harvested cells less than 4 passages were used for all experiments.

Proliferation Assay—Proliferation rates were measured through cell counting and proliferating cellular nuclear antigen (PCNA) Western blot analysis. 50,000 juvenile and adult osteoblasts were plated separately on tissue culture plates in triplicate. Cells were harvested each day post-plating and cell number was determined by counting with a hemacytometer. To determine the effects of FGF-2 on osteoblast proliferation, cell fractionation was analyzed by trypsinization and DNA (Clontech) at 68 °C for 1 h, followed by hybridization with [ω²-32P]dCTP labeled rat cDNA probe for 6 h at 68 °C. Stringency washes were performed with 1× sodium saline citrate (1× = 15 mM NaCl, 1.5 mM sodium citrate, pH 7) and 0.1× sodium dodecyl sulfate at room temperature for 10 min followed by 0.1× sodium saline citrate/0.1× sodium dodecyl sulfate at 68 °C for 20 min. Membranes were blocked in 20% sheep serum and then incubated with phosphorimaging plates overnight and analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Resulting images were quantified using ImageQuant image analysis software (Molecular Dynamics). Equal RNA loading and uniformity of blot transfer were assessed by stripping and reprobing the same membranes with a probe for β-actin. The same amount of DNA per lane was loaded for each time point in the experiment (35).

Western Blot Analysis—Juvenile and adult osteoblasts were grown to subconfluence then either harvested or stimulated with 10 ng/ml rhFGF-2 for 24 h. For analysis of receptor phosphorylation, cells were harvested with 10 ng/ml rhFGF-2 and 1 mg/ml sodium orthovanadate (Sigma, stock of 200 μM in water, pH 10) for 15 min before harvesting. Control cultures were serum-starved for 5 h and then incubated with phosphate-buffered saline. Cell lysate was harvested by incubating cells in radiolabeled precipitation assay buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitor mixture (Sigma-Aldrich) for 10 min at 4 °C and sheared with

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a syringe. Debris was removed by centrifugation for 5 min at 4 °C. For membrane preparation, cells were swelled in 20 mM Tris-HCl (pH 7.4) and homogenized with a Dounce homogenizer (Kontes, Vineland, NJ) (36). Briefly, nuclei were pelleted by centrifugation at 600 × g for 10 min. Remaining supernatant sheared with a syringe and further centrifuged at 20,000 × g for 20 min. The pelleted membrane associated proteins were then collected. Protein concentration was assessed with total RNA from each time point using oligo(dT)-priming in separate (BioRad).

and antibodies specific for goat were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary antibodies specific for mouse and rabbit were purchased from Amersham Biosciences. Juvenile osteoblasts showed uniform high level fluorescence compared with background.

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RESULTS

Extent of Maturation in Juvenile and Adult osteoblasts—Initial studies assessed states of differentiation in juvenile (2-day-old) and adult (60-day-old) rat calvarial osteoblasts based on properties of morphology and osteocalcin production. After 3 days in culture, freshly harvested juvenile and adult osteoblast cultures contained mixed populations of adherent cells; however, juvenile cultures contained a larger subpopulation of immature spindle-shaped cells, while adult cultures contained a larger subpopulation of mature cuboidal cells (Fig. 1A). Immunofluorescence for osteocalcin, a late marker of differentiation, demonstrated that adult osteoblast cultures showed uniform intense immunoreactivity for osteocalcin (91% positive), while juvenile osteoblast cultures had a mixed population of immunoreactive cells (57% positive) as compared with background fluorescence and nonspecific binding to control (NIH3T3) cells (Fig. 1B).

Proliferation and Attachment Rates—Differences in morphology and osteocalcin production suggested varied states of differentiation between juvenile and adult osteoblasts and led to investigation of proliferation and attachment rates. Juvenile osteoblasts proliferated significantly faster than adult osteoblasts on days 3–12 (Fig. 2A). This result may have been due to

| A | B | C |
|---|---|---|
| Juvenile | Adult | Control |
| osteoblasts produced equally small amounts of | osteoblasts produced equally small amounts of | osteoblasts produced equally small amounts of |
| integrin α2, integrin β1, and activated FAK. | integrin α2, integrin β1, and activated FAK. | integrin α2, integrin β1, and activated FAK. |

Fig. 1. Morphology and differentiation states of juvenile and adult osteoblasts. A, crystal violet staining demonstrated that juvenile osteoblasts contained less differentiated subpopulations (spindle-like) and adult osteoblasts contained more differentiated populations (cuboidal-shaped) at 40× magnification. B, osteocalcin immunofluorescence of juvenile osteoblasts, adult osteoblasts and control at ×10 magnification. Juvenile osteoblasts show uniform high level fluorescence compared with background.
Gene Expression in Response to rhFGF-2

Because of the reported differential effects of FGF-2 on osteoblasts of varied maturation, gene expression in juvenile and adult osteoblasts was assessed in response to FGF-2 stimulation. Northern blot analysis or real time PCR for collagen Iα1 (early differentiation/proliferation), osteopontin (intermediate differentiation/matrix maturation), and osteocalcin (late differentiation/matrix mineralization) was performed. At baseline levels, juvenile
osteoblasts expressed 3-fold more collagen Iα1 mRNA than adult osteoblasts (Fig. 4A). After 10 h of rhFGF-2 stimulation, juvenile osteoblasts increased their expression of collagen Iα1 5-fold, while the expression was comparatively unchanged in the adult osteoblasts. The expression of osteopontin in juvenile and adult cultures was equivalent at baseline levels; however, with rhFGF-2 stimulation juvenile osteoblasts increased their expression 13-fold by 24 h, while adult osteoblasts only increased their expression 5-fold (Fig. 4B). Finally, adult osteoblasts expressed 3-fold more osteocalcin than juvenile osteoblasts at baseline. After 3 days of rhFGF-2 stimulation, juvenile osteoblasts increased their osteocalcin expression nearly 16-fold, while adult osteoblast osteocalcin expression was relatively unchanged (Fig. 4C).

Bone Nodule Formation—As osteoblasts become fully differentiated, they increase their production and deposition of an organized collagen extracellular matrix. In vitro, the collagen extracellular matrix is mineralized to form bone nodules. After 4 weeks in differentiation media, young osteoblasts produced 41-fold more bone nodules than adult osteoblasts, as measured by bone nodule size and density (Fig. 4D).

Production, Autoregulation, and Modulation of FGF-2 Protein—To further understand the pathways involved in proliferation and differentiation, we examined juvenile versus adult osteoblast regulation of FGF-2 production and localization. At baseline, FGF-2 immunoreactivity demonstrated that juvenile osteoblasts produced less FGF-2 that localized mainly to the cytoplasm, while adult osteoblasts produced more FGF-2 that was found both in the cytoplasm and nucleus (Fig. 5A). After stimulation with rhFGF-2, juvenile osteoblasts increased their intracellular FGF-2 to resemble that of the adult cultures; FGF-2 was now localized to both the cytoplasm and nucleus of juvenile and adult osteoblasts.

Western blot analysis of juvenile and adult osteoblasts further demonstrated that juvenile osteoblasts produced less total FGF-2 protein than adult osteoblasts (Fig. 5B). Interestingly, juvenile osteoblasts translated equal levels of both the low and high molecular weight (LMW and HMW) forms of FGF-2, while adult osteoblasts preferentially translated an increased amount of the HMW (22 kDa) form of FGF-2. Upon rhFGF-2 (18 kDa) stimulation, both juvenile and adult osteoblasts showed an increase in intracellular FGF-2 protein, which resulted from both an uptake of exogenous FGF-2 and an increase in endogenous production of FGF-2. Evidence of exogenous FGF-2 uptake was identified by an increase of the 18 kDa band and the presence of a 16-kDa band, which represents the product of the internalized and partially cleaved 18-kDa form of FGF-2 as previously described (37). The increase in the high molecular weight forms suggests an autoregulation in FGF-2 translation. Interestingly, following rhFGF-2 stimulation juvenile osteoblasts switched from a low FGF-2 translation to an increased level of FGF-2 translation, mimicking the adult osteoblasts.

Furthermore, because of the modified phenotype (decreased proliferation and increased differentiation, data not shown) exhibited by juvenile osteoblasts after extended culture (2 months), juvenile and adult osteoblasts were analyzed for FGF-2 production. After extended culture, juvenile osteoblasts
began to modulate FGF-2 translation by specifically up-regulating HMW FGF-2 forms to mirror that of untreated adult osteoblasts, while the FGF-2 profile of adult osteoblasts resembled that of adult osteoblasts after FGF-2 stimulation (Fig. 5C). These results suggest that preferential expression of HMW FGF-2 assists in the more differentiated state.

FGF-2 is known to be an osteoblast mitogen. Western blot analysis of PCNA demonstrated that rhFGF-2 stimulation increased proliferation in both juvenile and adult osteoblasts (Fig. 5D). However, adult osteoblast PCNA levels after rhFGF-2 stimulation did not reach baseline levels of juvenile osteoblast PCNA production. These results suggested the higher proliferative state as well as the less differentiated state of juvenile osteoblasts.

FGF-R1 and -R2 mRNA, protein, and phosphorylation states. Expression of FGF-R1 and -R2 mRNA are believed to reflect osteoblast cellular differentiation and proliferation, respectively (23). Since our data indicated that juvenile osteoblasts proliferated significantly faster than adult osteoblasts, we investigated the regulation of FGF-R1 and FGF-R2 expression. At baseline levels, adult osteoblasts produced twice as much FGF-R1 mRNA than juvenile osteoblasts (Fig. 6A). However, after 24 h of rhFGF-2 stimulation, juvenile osteoblasts increased their expression of FGF-R1 mRNA 2.5-fold to equal that of adult osteoblasts, indicating that the young osteoblasts may have rapidly differentiated after 24 h of rhFGF-2 stimulation (Fig. 6A). Interestingly, baseline levels of FGF-R2 mRNA were more than 5-fold higher in juvenile osteoblasts compared with adult osteoblasts (Fig. 6B). In the presence of rhFGF-2, juvenile osteoblasts transiently increased their expression of FGF-R2 mRNA to almost 6.5-fold over the expression of adult FGF-R2 mRNA at 5 h and then decreased their expression by more than half at 24 h (Fig. 6B). These results support the reports by Iseki et al. (23) that FGF-2 bead-driven stimulation in fetal coronal sutures induced the adjacent skeletogenic membrane to increase expression of FGF-R1 and decrease expression of FGF-R2 by 24 h.

Because of the differential transcript levels, we further examined FGF-R1 and -R2 protein abundance and phosphorylation potential. Surprisingly, Western blot analysis demonstrated that juvenile osteoblasts translated 4-fold more FGF-R1 and 3-fold more FGF-R2 protein compared with adult osteoblasts (Fig. 6, C and D), indicating the increased potential for FGF-2 signaling in juvenile osteoblasts. After rhFGF-2 stimulation for 24 h, juvenile and adult osteoblasts did not significantly increase their production of FGF-R1 and -R2 protein, although adult osteoblast receptor levels after stimulation remained substantially reduced compared with juvenile osteoblast receptor baseline levels (Fig. 6, C and D).

Because of the large disparity in the abundance of FGF-R, we investigated the functionality of these receptors through their phosphorylation. FGF-R1 and -R2 were immunoprecipitated out of total membrane-associated protein and then immunoblotted for phosphorylation status. Juvenile osteoblasts showed increased phosphorylation for both FGF-R1 and -R2 protein after 15 min of rhFGF-2 stimulation (Fig. 6, C and D), indicating increased FGF-R1 and -R2 signaling. Whereas, in adult osteoblasts the FGF-2 signaling was minimal through FGF-R1 and almost absent through FGF-R2. Thus, these results clearly demonstrate the active FGF-2 signaling in juvenile, compared with adult, osteoblasts.

**DISCUSSION**

Infants successfully ossify large cranial defects; however, this regenerative capacity is lost in children older than 2 years of age. The molecular mechanisms underlying this regenerative capacity are still largely unknown (11). Most investigations have focused on the underlying dura mater; however, this study investigated the age-related cellular and molecular differences between juvenile and adult primary osteoblast cultures and has shown a correlation between their states of proliferation, differentiation, and osteogenic potential in vitro.

As demonstrated in previous studies, primary newborn mouse osteoblast cultures contained morphologically-mixed populations of spindle-shaped (immature) and cuboidal (mature) cells (12). We found that juvenile osteoblasts contained a larger number of immature cells, while adult osteoblast cultures included mostly mature cells. Furthermore, the abundance of osteocalcin, a late marker of differentiation, in adult osteoblast cultures suggested their uniform differentiation as opposed to the varied levels in juvenile osteoblast cultures. Differences in morphology and osteocalcin production support our hypothesis that juvenile osteoblast cultures contain a larger subpopulation of less differentiated cells.

Further investigation demonstrated that juvenile osteoblasts proliferated and attached to culture dishes significantly
faster than adult osteoblasts. This ability may reflect the large immature population found in juvenile osteoblast cultures and begins to explain their quick adaptation to a new environment. The decreased potential of adult osteoblasts to proliferate and attach may follow an inability to quickly organize their cytoskeletal and extracellular matrix molecules. Our data demonstrated a low production and organization of critical cytoskeletal and extracellular matrix molecules in adult osteoblasts, including fibronectin (initial attachment), F-actin (cytoskeleton), integrin α2β1 (structure and signaling), and vitronectin (attachment and migration). Previous studies have shown a correlation between the presence of integrin α2 and a positive regulation of osteogenesis (38), alluding to the juvenile osteoblasts’ increased osteogenic potential. Additionally, previous reports have demonstrated that expression levels of integrin αβ1 change during development (39), and, furthermore, integrin α2 knockout mice die perinatally, indicating the importance of this protein developmentally (40). Because integrin signaling occurs through a dimer composition of α and β subunits (41) or integrin-FAK complexes (42), differences in individual subunits may not reflect actual integrin function. Other proteins including focal adhesion kinases (FAK), integrin α2, and integrin β1 showed equivalent low levels in juvenile and adult osteoblasts and may not have played a significant role in their different phenotypes. FAK activation is an immediate downstream result of integrin β1 signaling (43), therefore, low levels of integrin β1 predict low FAK phosphorylation. Collectively, these data indicated that juvenile osteoblasts produced and organized critical cytoskeletal and extracellular matrix proteins necessary for increased attachment, proliferation, and signaling to a greater degree than adult osteoblasts.

Because of conflicting reports in the literature as to the differential mitogenic and osteogenic effects of FGF-2 on osteoblasts of varied ages (12, 20–24, 31, 44, 45), we further investigated juvenile versus adult osteoblast gene expression in response to FGF-2. As osteoblasts differentiate, they express collagen Iα1 (early), osteopontin (intermediate), and osteocalcin (late). Collagen Iα1 is the main component of the bony extracellular matrix and showed increased expression in juvenile osteoblasts both before and after rhFGF-2 stimulation. These data demonstrated that juvenile osteoblasts expressed more early markers of differentiation, were more responsive to rhFGF-2, and were primed to produce more bone. Osteopontin is associated with matrix formation and maturation. Juvenile and adult osteoblasts expressed equivalent baseline levels of osteopontin; however, after rhFGF-2 stimulation juvenile osteoblasts, in contrast to adult, dramatically increased their expression of osteopontin. Our findings agree with Iseki et al.; they found an increase of osteopontin expression after 24 h of FGF-2 stimulation in fetal mouse osteogenic progenitor cells in vitro (23). Finally we examined osteocalcin, a third extracellular protein thought to play a role in matrix mineralization. Previous studies have alluded to the low or absent expression of osteocalcin in osteoprogenitor cells in vivo as compared with in vitro (20). Consistently, osteocalcin expression was most detectable by the sensitive real time PCR analysis. At baseline levels, adult osteoblasts expressed more osteocalcin than juvenile osteoblasts, indicating that the adult osteoblasts maintained a differentiated phenotype. After rhFGF-2 stimulation, juvenile osteoblasts dramatically increased their osteocalcin expression, while adult osteoblasts did not alter their osteocalcin expression. Together, these results indi-
icated the increased potential of the juvenile osteoblasts to produce and mineralize bone.

Previous work has demonstrated the ability of osteoprogenitor cells to differentiate down the osteoblastic lineage in the presence of chemicals, including β-glycerophosphate, dexamethasone, and ascorbic acid (46). Thus, stimulation with osteoblast differentiation medium demonstrated increased matrix mineralization in juvenile osteoblasts through a 41-fold increase in bone nodule formation in vitro. These data further confirmed our results indicating that juvenile osteoblast cultures have a greater osteogenic potential and larger osteoprogenitor population than adult osteoblast cultures.

Because of the differential response rhFGF-2 stimulation, we further investigated endogenous FGF-2 production and regulation. FGF-2 is transcribed as a single mRNA, with at least 3 sites for initiation of translation (47, 48). FGF-2 forms have different roles depending on species, tissue type, developmental stage, and environmental conditions (49). The high molecular mass forms (HMW; 21.5 and 22 kDa) accumulate in the nucleus, while the low molecular mass form (LMW; 18 kDa) is mainly cytoplasmic, although it is poorly secreted (47, 50–52). Additionally, the HMW forms yield a different phenotype than the LMW form, indicating that HMW and LMW forms can have distinct targets and functionally roles (53, 54).

Interestingly, adult osteoblasts produced more total FGF-2, which was mainly composed of the HMW form, while juvenile osteoblasts produced low levels of all three forms. It is tempting to speculate that the preferential HMW FGF-2 translation in adult osteoblasts may contribute to their mature phenotype. Additionally, FGF-2 has a gradient effect on osteoblast proliferation and differentiation (23, 33, 55). Our results associated low levels of FGF-2 with juvenile osteoblasts into a more proliferative state, and high levels of FGF-2 with adult osteoblasts into a more differentiated state. Furthermore, rhFGF-2 stimulation of juvenile and adult osteoblasts increased both LMW and HMW FGF-2 production through both exogenous uptake and an autoregulation of endogenous FGF-2 production. Differential FGF-2 concentration and form production further support the idea of different role(s) for FGF-2 forms during regulation of development, proliferation, differentiation, and osteogenesis and may contribute to the phenotypic differences observed in juvenile and adult osteoblasts (54, 56, 57).

As primary osteoblasts were cultured for longer periods of time they began to lose their initial phenotypic and gene expression profile, so we further investigated FGF-2 production after extended culture (2 months). Juvenile osteoblasts showed a modulation in FGF-2 translation, whereby they preferentially up-regulated translation of the HMW FGF-2 form to resemble that of unstimulated adult osteoblasts. This indicated that extended culture influenced translational regulation of FGF-2 and induced juvenile osteoblasts to become more differentiated corresponding to a modified phenotype.

Because of the known mitogenic effects of FGF-2, we investigated juvenile versus adult osteoblast proliferation rates in response to rhFGF-2 stimulation. Both juvenile and adult osteoblasts increased proliferation upon rhFGF-2 stimulation, although juvenile osteoblasts consistently proliferated faster. These data, in combination with the gene expression data, indicated that juvenile osteoblasts were more responsive to the mitogenic and osteogenic effects of FGF-2.

Fibroblast growth factor-2 elicits a signal when bound to high affinity FGF receptors (FGF-R 1–4). FGF-R1 mRNA marks differentiated osteoblasts while FGF-R2 mRNA marks proliferative osteoblasts; both are expressed by osteoblasts of the parietal bone (23) and bind FGF-2 with equal affinity (34). Thus, regulation of FGF-2 signaling must occur not only with ligand availability, but also with receptor abundance and availability. Osteoblast proliferation and differentiation were further investigated through FGF-R production and signaling. Consistent with previous reports, adult osteoblasts transcribed more FGF-R1, while juvenile osteoblasts transcribed more FGF-R2 at baseline levels. Upon rhFGF-2 stimulation, juvenile osteoblasts first temporarily increased their expression of FGF-R2 after which they increased FGF-R1 expression, indicating their transition from a proliferative to a differentiated state. In contrast, rhFGF-2 stimulated adult osteoblasts maintained FGF-R1 expression and only slightly increased expression of FGF-R2 after 24 h. Accordingly, Iseki et al. demonstrated in vivo that osteogenic stem cells of fetal mice calvaria stopped proliferating 6 h after stimulation with rhFGF-2-soaked beads (23). In addition, this FGF-2 bead-driven stimulation in fetal coronal sutures induced the adjacent skeletal membrane to increase expression of FGF-R1 and decrease expression of FGF-R2 by 24 h. While the work of Iseki et al. was performed in vivo on a mixed “osteogenic stem cell” population in the embryonic calvaria, our work clearly demonstrated the shift in FGF-R1 to -R2 expression of osteoblasts in vitro.

Because mRNA transcription is not always an accurate reflection of protein production, we further investigated FGF-R translation in juvenile and adult osteoblasts. The data demonstrated that juvenile osteoblasts translated more FGF-R1 and -R2 protein at baseline levels. After rhFGF-2 stimulation, juvenile and adult osteoblasts did not significantly increase their production of FGF-R1 and -R2. These data are important because FGF-R1 and -R2 protein perform the signaling function and do not necessarily reflect the mRNA-associated states of proliferation or differentiation, but instead may reflect differential signaling pathways. Thus, juvenile osteoblasts had an increased amount of receptor potentially available for binding to FGF-2, while adult osteoblasts had minimal receptor amounts.

Finally, because subtle differences in FGF-2 signaling may profoundly affect phenotype (33), the signaling potential of FGF-R1 and -R2 was determined by analyzing phosphorylation states. Upon 15 min of rhFGF-2 stimulation, juvenile osteoblasts phosphorylated more FGF-R1 and -R2 as compared with adult osteoblasts. This indicated the increased ability of juvenile osteoblasts to transmit signals through FGF-R1 and -R2 and thus increase their proliferation and then differentiation. Additionally, adult osteoblasts showed some phosphorylation of FGF-R1, but almost an absence of FGF-R2 phosphorylation. Thus, expression of and signaling through FGF-R1 may lead to a differentiated phenotype, while that of FGF-R2 may lead to a proliferative phenotype. These results indicate that the adult osteoblasts may be in an almost quiescent state because of the reduced ability of their receptors to transmit a signal.

Previous research has demonstrated that states of proliferation are partially regulated by translation of different FGF-2 forms (52, 54), concentration of FGF-2, and receptor regulation (23). The present study indicated that juvenile osteoblasts were in a less differentiated state, attached and proliferated faster, translated low levels of HMW FGF-2, had less endogenous FGF-2, and had more cell surface associated FGF-R1 and -R2 protein with increased phosphorylation. In contrast, adult osteoblasts were in a fully differentiated state, preferentially increased translation of HMW FGF-2, produced high levels of FGF-2, and demonstrated less FGF-R1 and -R2 protein and phosphorylation. Thus, the large subpopulation of less differentiated osteoblasts found in juvenile calvaria are more responsive to FGF-2 signaling, while osteoblasts of adult calvaria are minimally responsive leading to “locked out” FGF-2 signaling.
In summary, we hypothesize that juvenile calvaria contain a subpopulation of pre-osteoblasts in a low FGF-2 environment, which are capable of proliferating and migrating into the area of a skull defect. At this point, pre-osteoblasts encounter a high FGF-2 environment supplied by the juvenile dura mater underlying the calvarial defect (26). Pre-osteoblasts differentiate and mineralize a bony matrix mediating successful calvarial ossification. Since adult osteoblasts demonstrate low proliferation, low FGF-R1 and -R2 signaling, and minimal ability to form bone nodules, they cannot participate in successful healing. These results deserve further investigation into the roles of the LMW and HMW forms of FGF-2 in juvenile versus adult osteoblasts as well as the gene expression and phenotype resulting from either FGF-R1 or -R2 signaling.

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Additions and Corrections

Vol. 278 (2003) 22136–22143

Novel localization of the DNA-PK complex in lipid rafts. A putative role in the signal transduction pathway of the ionizing radiation response.

Hector Lucero, Darren Gae, and Guillermo E. Taccioli

Page 22136: The following grant statement should be included: "Dr. Lucero is supported by National Institutes of Health Grant GM31318."

Vol. 278 (2003) 32005–32013

Age-related changes in the biomolecular mechanisms of calvarial osteoblast biology affect fibroblast growth factor-2 signaling and osteogenesis.

Catherine M. Cowan, Natalina Quarto, Stephen M. Warren, Ali Salim, and Michael T. Longaker

The word “calvarial” was misspelled. The corrected title is shown above.

Vol. 278 (2003) 36959–36965

CCAAT/enhancer-binding protein family members recruit the coactivator CREB-binding protein and trigger its phosphorylation.

Krisztian A. Kovacs, Myriam Steinmann, Pierre J. Magistretti, Olivier Halfon, and Jean-Rene Cardinaux

Page 36965, line 3: The sentence should read, “Similarly, the cyclin E-Cdk2 complex, which may target the same sites as MAPK, phosphorylates CBP in a cell cycle-dependent manner, thus enhancing its HAT activity (71).”

Vol. 278 (2003) 40425–40428

YXXM motifs in the PDGF-β receptor serve dual roles as phosphoinositide 3-kinase binding motifs and tyrosine-based endocytic sorting signals.

Haiyan Wu, David A. Windmiller, Ling Wang, and Jonathan M. Backer

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We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.