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TGF-β1 Calcium Signaling in Osteoblasts

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Abstract Transforming growth factor-β1 (TGF-β1) action is known to be initiated by its binding to multiple cell surface receptors containing serine/threonine kinase domains that act to stimulate a cascade of signaling events in a variety of cell types. We have previously shown that TGF-β1 and BMP-2 treatment of primary human osteoblasts (HOBs) enhances cell-substrate adhesion. In this report, we demonstrate that TGF-β1 elicits a rapid, transient, and oscillatory rise in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, that is necessary for enhancement of cell adhesion in HOBs but does not alter the phosphorylation state of Smad proteins. This rise in $[Ca^{2+}]_i$ in HOB is not observed in the absence of extracellular calcium or when the cells are treated with the L-type Ca^{2+} channel blocker, nifedipine, but is stimulated upon treatment with the L-type Ca^{2+} channel agonist, Bay K 8644, or under high K^+ conditions. The rise in $[Ca^{2+}]_i$ is severely attenuated after treatment of the cells with thapsigargin, a selective endoplasmic reticulum Ca^{2+} pump inhibitor. TGF-β1 enhancement of HOB adhesion to tissue culture polystyrene is also inhibited in cells treated with nifedipine. These data suggest that intracellular Ca^{2+} signaling is an important second messenger of the TGF-β1 signal transduction pathway in osteoblast function. J. Cell. Biochem. 101: 348–359, 2007.

Key words: TGF-β1; Ca^{2+} signaling; osteoblast; cell adhesion; Ca^{2+} channel; Smad; Fura-2

Transforming growth factor-β1 (TGF-β1) is a multifunctional signaling protein that initiates a wide variety of responses in many different cell types. For example, TGF-β1 action is involved in embryogenesis, differentiation, promotion of wound healing, fracture healing, extracellular matrix (ECM) production, and cell adhesion [Centrella et al., 1994; Alevizopoulos and Mermod, 1997; Shah et al., 1997; Schmidmaier et al., 2004; Wildemann et al., 2004; Thapa et al., 2005]. Interestingly, the mineralized bone matrix is one of the richest depots of TGF-β1 in the body. Osteoblasts contain a large number of high affinity TGF-β1 receptors, and TGF-β1 regulates many osteoblast functions, including the expression of ECM genes, such as collagen type I and fibronectin, and their integrin receptors, as well as stabilization of integrin subunits [Ignotz and Massague, 1987; Robey et al., 1987; Dean et al., 1988; Roberts et al., 1988; Heino et al., 1989; Harris et al., 1994; Dallas et al., 2005; Lee et al., 2006].

Our laboratory has previously shown that adhesion of primary human osteoblasts (HOBs) to substrates, including biomaterials used for

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orthopedic implants, is enhanced by pretreatment with TGF-β1 or BMP-2 [Shah et al., 1999]. Osseointegration, the close apposition of bone to adjacent biomaterials [Branemark et al., 1977], is believed to be critically dependent on optimal osteoblast adhesion and proliferation, bone matrix production, and mineralization at the bone-implant interface [Sinha et al., 1994]. Cell adhesion is the first crucial step for osseointegration, as it essentially specifies the spatial fate of the cell. In addition, cell proliferation, differentiation, ECM production, and organization, as well as apoptosis have all been shown to be influenced by the events of cell-substrate adhesion [Sinha et al., 1991; Frisch et al., 1996; Sastry and Horwitz, 1996; Harbers and Healy, 2005; Advincula et al., 2006; El-Amin et al., 2006]. Thus, enhancing the adhesive activities of osteoblasts may not only improve their ability to "grow into" the surface architecture of biomaterials and promote the longevity of osseous implants, but also improve their functional interaction with the existing bone matrix.

Two types of TGF-β1 receptors (TβRs) are generally believed to initiate the signal transduction cascade [Hill, 1996; Alevizopoulos and Mermod, 1997; Heldin et al., 1997; Huang and Huang, 2005; Faler et al., 2006]. The TGF-β1 receptor complex consists of two type II (TβRII) and two type I (TβRI) receptors. TGF-β1 initially binds to a constitutively active serine/threonine kinase specific for TβRI. This ligand-receptor complex then recruits and phosphorylates TβRI, thereby activating the TβRI kinase to form a heterotetrameric TGF-β receptor complex which initiates signal transduction.

The downstream signaling cascade for TGF-β1 has been shown to consist of many pathways and components. Much of the literature has dealt with receptor associated Smad proteins as the primary mediators of TGF-β1 signaling. Whether the Smad pathway is the dominant downstream signaling cascade after TGF-β1-receptor interaction remains to be established [Oide and Thurman, 1994; Ishiyama et al., 1996; Alevizopoulos et al., 1997; Javelaud and Mauviel, 2005; Yoshida et al., 2005]. Here, we report a TGF-β1 induced intracellular Ca^{2+} flux in HOB, a result of the opening of nifedipine-sensitive Ca^{2+} channels. The resulting change in intracellular Ca^{2+} level, $[Ca^{2+}]_{i,}$ is necessary for the TGF-β1 mediated enhancement of HOB adhesion to substrate.

MATERIALS AND METHODS

Cell Preparation

All in vitro experiments described were carried out on cultured primary HOB, isolated using the protocol of Robey and Termine [1985], modified by Sinha and Tuan [1996]. Briefly, bone chips from human trabecular bone were digested with collagenase P (Sigma), plated in calcium free DMEM/F12K medium, supplemented with 10% fetal bovine serum (Atlanta Biological), 2 mM L-glutamine, 25 μg/ml ascorbate, and 50 μg/ml penicillin–streptomycin, and incubated at 37°C in 7.5% CO_2. Cells migrated from the bone chips within 10–14 days and reached confluency within 4 weeks. The cultures were then switched to medium containing a normal level of calcium (1.1 mM), referred to as standard medium. The cells were used for experiments between 4 and 10 weeks of culture prior to the second passage. All procedures using human tissue were approved by the Institutional Review Board of Thomas Jefferson University.

Measurement of Intracellular Ca^{2+}

TGF-β1 induced $[Ca^{2+}]_i$ response was measured in Fura-2 loaded HOB by fluorescence imaging [Tsien, 1989] using a previously described setup [Rooney et al., 1989]. HOB were plated at a density of 10,000 cells/cm^2 on No. 1 glass coverslips and loaded with the Ca^{2+} indicator, Fura-2, by incubation with 10 μM Fura-2/AM (Molecular Probes) for 20 min at 37°C. A modified Hank's Balanced Salt Solution (HBSS), consisting of 121 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM glucose, and 0.18 mg/ml bovine serum albumin (Sigma), pH 7.4, was used for all fluorescence imaging experiments. The $[Ca^{2+}]_i$ response to TGF-β1 was measured in individual cells within populations using a fluorescence imaging microscope (Olympus) equipped with a cooled CCD camera (Photometrics). After washing, cells were mounted on a temperature controlled microscope stage that provided easy access for medium changes. The temperature was held constant at 37°C for all imaging experiments. Wavelengths used were: excitation, 340 and 380 nm; and emission, 510 nm. An experimental run typically included 204 image sets taken at 5 s intervals with 200 ms shutter speed. Baseline $[Ca^{2+}]_i$ levels were measured

for 2 min, then recombinant human TGF-β1 (10 ng/ml; R&D Systems) was added, and $[Ca^{2+}]_i$ was measured for an additional 15 min. In some experiments, nifedipine (10 μM, Sigma) or Bay K 8644 (10 μM, Biomol) was added as antagonist and agonist of L-type Ca^{2+} channels, respectively. Thapsigargin (10 μM, Sigma) was also used to some experiments to block the endoplasmic reticulum Ca^{2+} pump.

Cell Adhesion Assay

HOB adhesion to substrate was assayed as described previously [Sinha and Tuan, 1996; Shah et al., 1999]. HOB were plated on 24 well (1.9 cm^2/well) tissue culture polystyrene plates (Costar) at a density of 10,000 cells/cm^2, and then incubated for 12 h at 37°C in the presence of standard medium, medium supplemented with TGF-β1 (10 ng/ml), medium supplemented with nifedipine (10 μM), or medium supplemented with both TGF-β1 and nifedipine at the same respective concentrations. At 3, 6, and 12 h time points, non-adherent cells were rinsed away with phosphate buffered saline (PBS), pH 7.4, and adherent cells were labeled for 30 min with the fluorescent dye, BCECF-AM (10 mM, Molecular Probes) [Wierda et al., 1989]. The labeled cells were then lysed with 1% Triton X-100 and cell number was estimated by measuring the released dye by spectrofluorometry (excitation, 485 nm; emission, 535 nm).

Smad-2 Phosphorylation Assay

Cell lysate preparation. HOB plated at a density of 5×10^5 cells per 25 cm^2 vented tissue culture flask (Corning) and cultured for 7 days were stimulated for 15 min at 37°C as follows: untreated control, 10 ng/ml of TGF-β1, 10 ng/ml of TGF-β1 and 10 μM nifedipine, and 10 μM nifedipine alone. After washing with ice-cold PBS, cells were lysed by shaking for 15 min at 4°C with 800 μl of protein extraction buffer, consisting of 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin), 1 mM activated Na_3VO_4, and 1 mM NaF. Cell lysate was collected as the supernatant after centrifugation.

Immunoprecipitation. Cell lysates were precleared by incubation with Protein G agarose beads (Gibco), and protein concentration determined using the BCA micro assay (Pierce). To 3 mg of HOB protein extract, a Smad-2 antibody generated in mouse (Santa Cruz), was added at a dilution of 1:1,000, and the cell lysate/antibody mixture incubated with shaking at 4°C overnight. The immunocomplex was captured by the addition of Protein G beads, washed in ice-cold PBS, and solubilized by boiling for 5 min in 2× SDS–PAGE sample buffer (BioRad).

Western blot analysis. Immunoprecipitates were separated by SDS–PAGE on a 7.5% polyacrylamide gel (BioRad) and electrotransferred to nitrocellulose paper (Hybond). After blocking with 5% bovine serum albumin, the blot was incubated with either anti-phosphoserine or anti-Smad-2 antibodies (Santa Cruz, 1:1,000). Immunoreactive protein was detected by HRP-chemiluminescence (NEN Life Sciences).

RESULTS

Our laboratory has previously shown that a 12-h pretreatment with TGF-β1 or BMP-2 resulted in an enhancement of HOB adhesion [Shah et al., 1999; Noth et al., 2003] (unpublished data). This enhanced adhesion is seen on both tissue culture polystyrene and metallic alloy biomaterials. The aim of this study is to examine the intracellular signaling pathways responsible for TGF-β1 enhancement of HOB adhesion.

TGF-β1 caused an immediate but transient change in $[Ca^{2+}]_i$ in HOB (Fig. 1). We monitored this effect in Fura-2 loaded primary HOB immediately after the addition of 10 ng/ml of TGF-β1. Greater than 70% of cells responded to the addition of TGF-β1 with an initial rise in $[Ca^{2+}]_i$, followed by oscillatory changes in $[Ca^{2+}]_i$. The initial response occurred immediately in almost all cells (Fig. 1A), and consisted of both single responses (Fig. 1B) and oscillatory responses (Fig. 1C,D). The average maximal $[Ca^{2+}]_i$ for responding cells was 222 nM. Subsequent oscillations were approximately half the magnitude of the initial peak and averaged 117 nM. Oscillations occurred at a frequency of 0.24 spikes per minute during the 15 min sampling period. There were few, if any, responses in the nifedipine treated group. Nifedipine treated cells that did respond showed average maximum $[Ca^{2+}]_i$ of 90.9 nM, and oscillatory spikes were approximately the same magnitude at 81.8 nM. Neither of these values was statistically different from baseline $[Ca^{2+}]_i$ levels. These data are summarized in Table I.

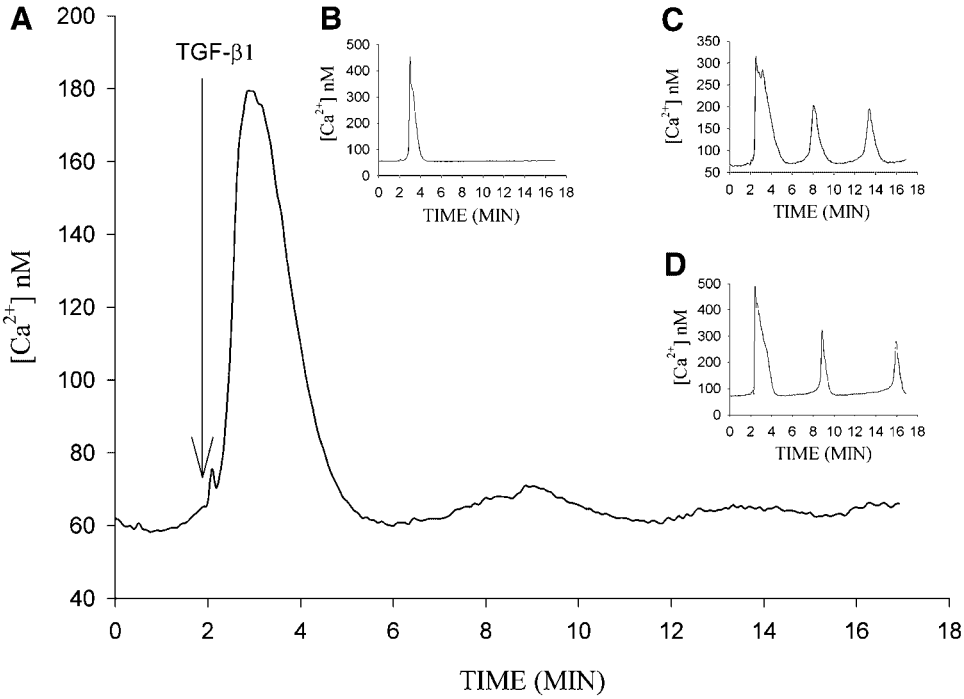

Fig. 1. TGF-β1 treatment of HOB results in transient rise in $[Ca^{2+}]_i$. HOB were isolated, cultured and treated with 10 ng/ml of TGF-β1 as described in Materials and Methods, and monitored for $[Ca^{2+}]_i$ using Fura-2 ratiometric spectrofluorometry. The results presented in the main figure (**A**) are an average from a population of cells (n ≈ 75). Representative single cell responses include single spike (**B**) and initial spike with subsequent $[Ca^{2+}]_i$ oscillations (**C**, **D**). All Ca^{2+} imaging experiments were repeated at least three times.

The importance of mechanical stimulation, including fluid shear, in the activation of osteoblast Ca^{2+} channels has been well documented [Duncan et al., 1998]. For this reason, great care was taken to prevent fluid shear while adding growth factor or any other experimental agonist or antagonist. Addition of imaging buffer alone (without growth factor) failed to elicit $[Ca^{2+}]_i$ transients in the tested cell populations.

We next examined whether the TGF-β1 induced rise in $[Ca^{2+}]_i$ was dependent on extracellular calcium (Fig. 2). Fura-2 loaded HOB were first placed in a calcium-free modified HBSS for 5–10 min before TGF-β1 stimulation and $[Ca^{2+}]_i$ monitoring. As shown in Figure 2, TGF-β1 treatment did not cause a rise in HOB $[Ca^{2+}]_i$ in the absence of extracellular calcium. That this failure to respond was not due to a depletion of intracellular calcium stores was shown by subsequently treating the HOB with 10 μM thapsigargin, a specific endoplasmic reticulum Ca^{2+} pump inhibitor, which resulted in an immediate release of stored intracellular calcium (Fig. 3). The TGF-β1 $[Ca^{2+}]_i$ response was restored with an attenuated response after

TABLE I. Effect of TGF-β1 Treatment on $[Ca^{2+}]_i$ in HOB†

	TGF-β	TGF-β1 and nifedipine	Sequential thapsigargin and TGF-β1 treatment
Baseline $[Ca^{2+}]$ (nM)	59.8 (±25.6)	56.4 (±39.2)	89.8 (±3.3)
Maximum rise in $[Ca^{2+}]_i$ (nM)	222.4 (±79.9)**	90.9 (±106.5)*	202.4 (±61.3)***
Oscillatory rise in $[Ca^{2+}]_i$ (nM)	117.3 (±56.4)$^{++}$	81.1 (±71.8)$^{+}$	100.4 (±7.7)$^{+++}$
Oscillations per minute	0.24	0	0
% Cells responding	75.8	0	Not determined

Maximum $[Ca^{2+}]_i$ versus baseline: $^{*}P = 0.02$; $^{**}P < 0.001$; $^{***}P < 0.005$.
Maximum $[Ca^{2+}]_i$ versus oscillation: $^{+}P = 0.56$; $^{++}P < 0.001$; $^{+++}P < 0.005$.
†n ≈ 240 for all analysis. All Ca^{2+} imaging experiments were repeated at least three times.

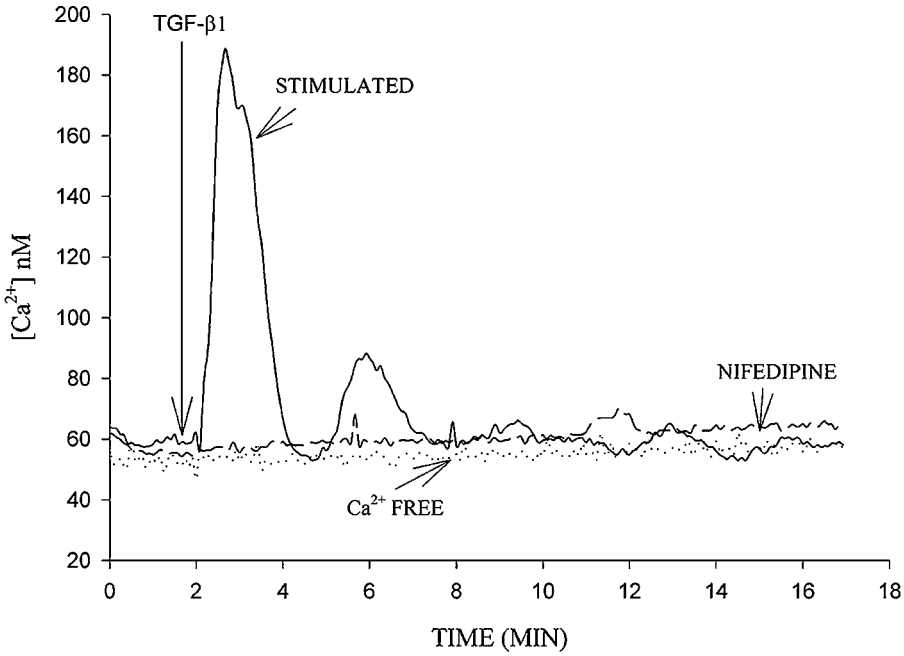

Fig. 2. TGF-β1 stimulation of $[Ca^{2+}]_i$ flux in HOB requires extracellular calcium and functional L-type Ca^{2+} channels. TGF-β1 treatment resulted in no significant change in $[Ca^{2+}]_i$ in HOB maintained in calcium-free medium or upon treatment with nifedipine. All Ca^{2+} imaging studies were repeated at least three times; $n \approx 75$ cells.

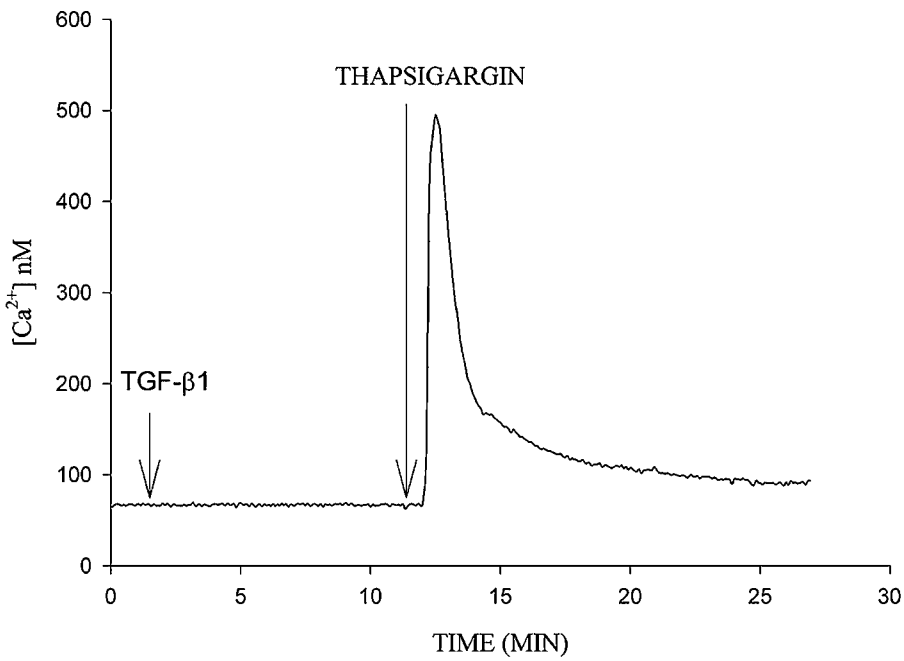

Fig. 3. TGF-β1 induced $[Ca^{2+}]_i$ flux requires extracellular Ca^{2+} influx for release of intracellular Ca^{2+} stores. HOB, in a calcium-free buffer solution, were treated first with TGF-β1 and then treated with thapsigargin to release intracellular Ca^{2+} stores. The results here demonstrate the relationship between TGF-β1 stimulated $[Ca^{2+}]_i$ flux, and extracellular Ca^{2+} influx and the release of intracellular Ca^{2+} stores. These Ca^{2+} stores are not depleted in the calcium-free buffer solution. All $[Ca^{2+}]_i$ imaging studies were repeated at least three times: $n \approx 75$ cells.

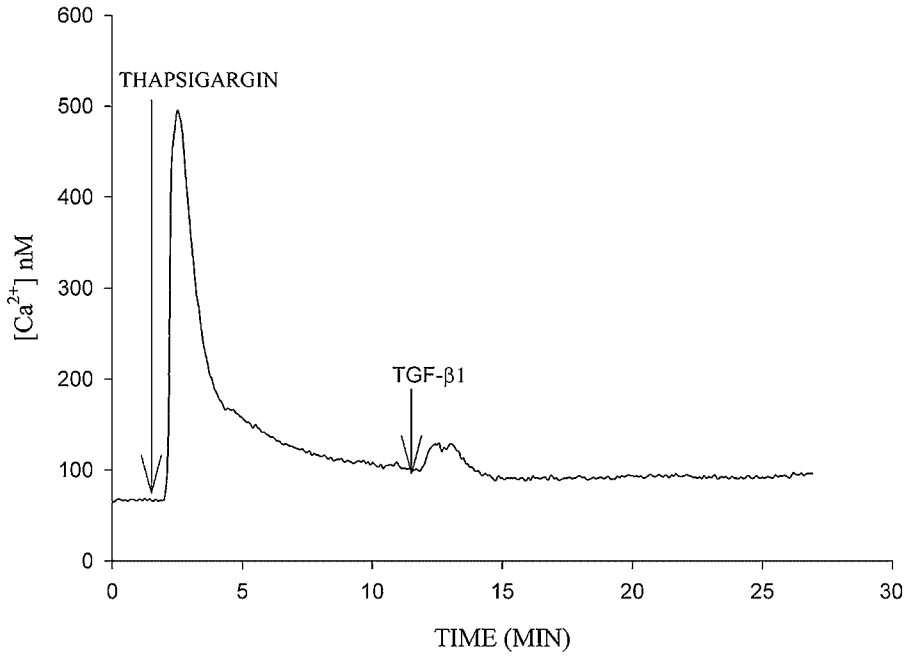

Fig. 4. TGF-β1 induced $[Ca^{2+}]_i$ flux requires intracellular calcium stores. As described in Materials and Methods, HOB were treated first with thapsigargin to release intracellular Ca^{2+} and then stimulated with TGF-β1. The severely attenuated response to TGF-β1 indicates the requirement of intracellular calcium stores for TGF-β1 induced Ca^{2+} signaling. All Ca^{2+} imaging studies were repeated at least three times: $n \approx 75$ cells.

replacement of extracellular calcium to the bathing medium (Fig. 4).

L-Type Ca^{2+} channels are characterized, in part, by their sensitivity to dihydropyridines (DHP) [Tsien et al., 1987]. HOB treated with 10 μM nifedipine, a selective L-type Ca^{2+} channel blocker, either failed to respond or showed an attenuated response after the addition of TGF-β1 (Fig. 2). On average, the normal initial TGF-β1 response increased $[Ca^{2+}]_i$ from a resting level of approximately 50 nM to an average stimulated level of 222 nM. Population studies and single cell monitoring demonstrated no response in cells pretreated with nifedipine (Fig. 2). To further characterize the HOB Ca^{2+} channel sensitivity to DHP, the cells were treated with 10 μM Bay K 8644, a selective L-type Ca^{2+} channel agonist. HOB responded with an increase in $[Ca^{2+}]_i$ that was similar to the TGF-β1 induced $[Ca^{2+}]_i$ response shown in Figure 1 (data not shown). Both single responses and oscillatory responses were seen. These results, therefore, suggest that TGF-β1 induced a change in intracellular Ca^{2+} metabolism, that is, initiated by the influx of extracellular Ca^{2+} through DHP-sensitive Ca^{2+} channels.

To test the voltage sensitivity of the HOB Ca^{2+} channels, the cells were placed in a high K^+ environment, 50 mM, to depolarize the cell membrane. A rise in $[Ca^{2+}]_i$ was observed (data not shown), consistent with the involvement of L-type Ca^{2+} channels which are classified as high voltage activated (HVA) voltage sensitive Ca^{2+} channels (VSCC). Both single responses and oscillatory responses were seen.

To determine the importance of intracellular calcium stores in the Ca^{2+} signaling response, HOB were treated sequentially with thapsigargin and TGF-β1. All cells released their intracellular calcium stores after thapsigargin treatment. Ten minutes after exposure to thapsigargin, TGF-β1 stimulation failed to elicit its characteristic response. In the responding cells, there was a severely attenuated rise in the $[Ca^{2+}]_i$ levels and no subsequent oscillations (Fig. 4). These results indicate that intracellular calcium stores are required for a complete TGF-β1 induced $[Ca^{2+}]_i$ response in the HOB. Thus, intracellular Ca^{2+} stores and extracellular Ca^{2+} influx worked in concert to significantly alter resting $[Ca^{2+}]_i$ and trigger $[Ca^{2+}]_i$ oscillations after TGF-β1 treatment of HOB.

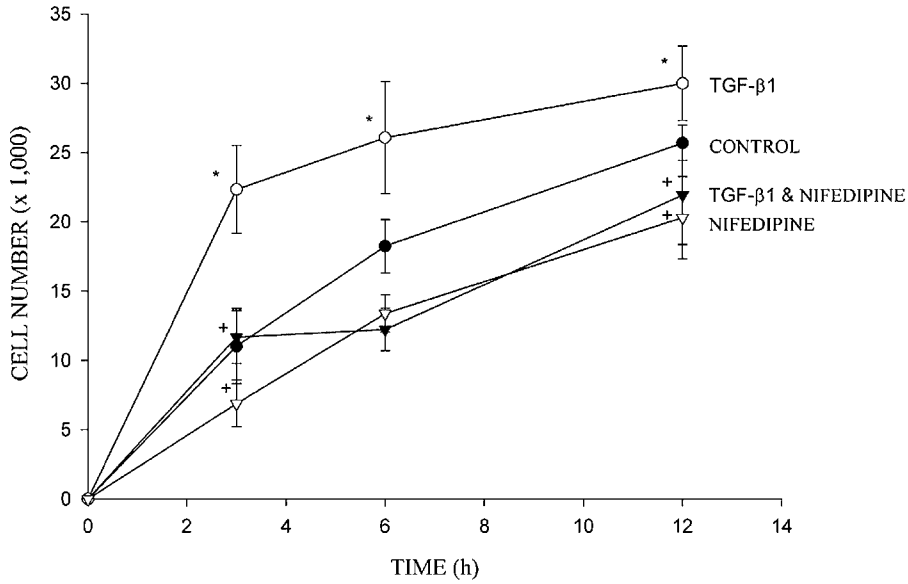

Fig. 5. TGF-β1 enhancement of HOB adhesion to culture substrate is dependent on $[Ca^{2+}]_i$ signal mediated by L-type Ca^{2+} channel. Compared to controls: $^*P = <0.05$; $^+P > 0.05$; n ≈ 75 cells. All samples were analyzed in triplicates and the experiment was repeated three times. All results are presented as mean ± SD.

Our results also showed that TGF-β1 induced Ca^{2+} influx was important for the enhancement of HOB adhesion (Fig. 5). Groups of HOB were pretreated for 12 h with 10 ng/ml TGF-β1, 10 μM nifedipine, or 10 ng/ml TGF-β1 and 10 μM nifedipine, and then assayed for adhesion activity as described in Materials and Methods. HOB not treated with supplemental growth factor or Ca^{2+} channel blocker served as the control group. As observed previously in similar assays [Faler et al., 2006], the number of adherent cells increased as a function of time with maximal adhesion level occurring at the 12-h time point. Cells treated with nifedipine showed minimal changes in initial adhesion kinetics and maximal adhesion level compared to control groups, indicating that nifedipine alone does not affect HOB adhesion without supplementation of growth factors. Cells pretreated with TGF-β1 for 12 h demonstrated a significantly increased rate of adhesion and overall level of adhesion compared to control groups ($P < 0.05$) as reported previously [Duncan et al., 1998]. Cells treated with nifedipine and then stimulated with TGF-β1 failed to exhibit the same enhanced adhesion kinetics and overall adhesion. These results suggest that TGF-β1 induced Ca^{2+} influx, mediated by nifedipine sensitive Ca^{2+} channels, is important for TGF-β1 enhancement of adhesion kinetics and overall adhesion.

To investigate the relationship between extracellular Ca^{2+} influx and Smad-2 phosphorylation, HOB were stimulated with TGF-β1 and/or nifedipine for 15 min and then lysed. After immunoprecipitation from total protein extract, Smad-2 was analyzed for its phosphorylation state by western blot, probed with an anti-phosphoserine antibody. TGF-β1 stimulation resulted in increased serine phosphorylation of Smad-2. However, phosphorylation was not affected by Ca^{2+} influx (Fig. 6a). Probing the western blot with Smad-2 antibody demonstrated equal levels of Smad-2 protein in all test groups (Fig. 6b). These results suggest that the TGF-β1 induced Ca^{2+} signal is not required for Smad-2 serine phosphorylation.

DISCUSSION

We have previously observed that TGF-β1 family member treatment of HOB enhanced their adhesion characteristics [Shah et al., 1999; Noth et al., 2003]. We identified cell spreading, increased focal contact formation, and increased ECM production as probable events modified by TGF-β1 and involved in adhesion enhancement. However, little is known about the signal transduction pathway responsible for these changes. Much of the attention in TGF-β1 signal transduction has been focused on the Smad proteins [Verschueren and

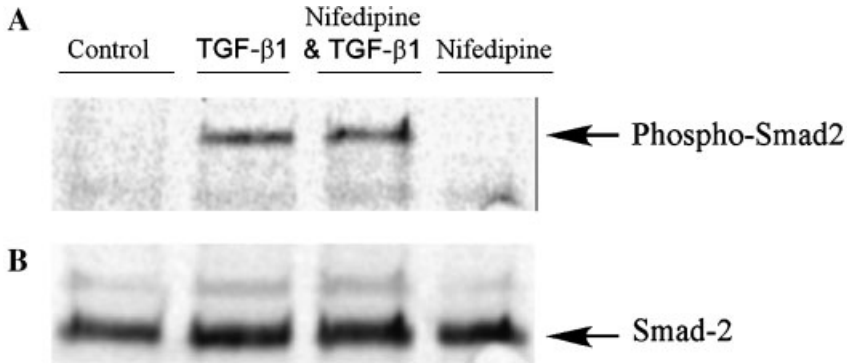

Fig. 6. Relationship between the TGF-β1 Ca^{2+} signal and Smad 2 phosphorylation. Protein extracts from HOB treated with TGF-β1 for 15 min were immunoprecipitated with Smad 2 antibodies and analyzed by Western immunoblotting. **A**: Immunoblot probed with anti-phosphoserine antibody. TGF-β1 treatment results in Smad 2 phosphorylation. Nifedipine does not affect TGF-β1 induced Smad 2 phosphorylation. **B**: Immunoblot probed with Smad 2 antibody demonstrating equivalent Smad 2 load in all samples.

Huylebroeck, 1999; Blobe et al., 2000; Javelaud and Mauviel, 2005; Hill, 2006]. In the TGF-β signaling pathway, activated TβRI phosphorylates Smads 2 and 3, which then complex with Smad 4 and translocate to the nucleus where they affect gene transcription. The results reported here show that TGF-β1 treatment causes an immediate but transient change in $[Ca^{2+}]_i$ in cultured HOB. Furthermore, this change in $[Ca^{2+}]_i$ is required for osteoblast adhesion enhancement, but it is not required for Smad-2 phosphorylation. Together, these data strongly suggest that the intracellular Ca^{2+}signal is functionally important for TGF-β1 signal transduction in osteoblasts.

The oscillatory changes in $[Ca^{2+}]_i$ are initiated by Ca^{2+} influx through a yet to be defined Ca^{2+} channel. Several studies have shown osteoblasts to express predominantly L-type Ca^{2+} channels and have demonstrated their importance in signal transduction events [Loza et al., 1994; Barry et al., 1995; Wang et al., 2004; Shao et al., 2005; Zahanich et al., 2005]. The channels responsible for TGF-β1 induced Ca^{2+} influx in HOB appear to have properties similar to those of an L-type Ca^{2+} channel, as shown by their sensitivity to DHP's and to increases in voltage. However, the nature of channel control has not yet been determined. Nevertheless, this initial Ca^{2+} influx after TGF-β1 treatment appears to induce intracellular Ca^{2+} release that results in $[Ca^{2+}]_i$ oscillations, suggestive of a maintenance signal, and contributes to adhesion enhancement. It appears that Ca^{2+} influx is crucial for intracellular Ca^{2+} release, as there is no response when cell membrane Ca^{2+} channels are blocked or when cells are stimulated in the absence of extracellular calcium. The exact mechanisms responsible for the closely linked extracellular Ca^{2+} influx and intracellular Ca^{2+} release remain to be determined.

Nifedipine-treated HOB do not show the enhanced adhesion properties in response to TGF-β1. In fact, the adhesion kinetics of nifedipine-treated cells, with or without the addition of TGF-β1, is similar to that of the untreated control group. This result shows that nifedipine itself has only a minimal negative effect on osteoblast adhesion in the absence of any additional growth factor. However, when nifedipine is used to block the TGF-β1 induced extracellular Ca^{2+} influx, adhesion enhancement is not seen, strongly suggesting the importance of extracellular Ca^{2+} influx in the TGF-β1 adhesion promoting effect.

The preponderance of the literature describes receptor associated Smad proteins as central to TGF-β1 signal transduction. TβRI directly serine phosphorylates Smad 2 which confers downstream effects [Heldin et al., 1997]. Here, we find that blocking TGF-β1 Ca^{2+} influx does not alter Smad-2 phosphorylation. This result suggests that there is a degree of separation between the Smad signaling pathway and TGF-β1 induced Ca^{2+} signaling pathway. Whether the separation exists upstream or downstream of Smad-2 phosphorylation remains to be determined. Understanding the importance of Smad-2 phosphorylation on Ca^{2+} channel opening and the significance of Ca^{2+} influx on downstream targets of Smad-2 will further elucidate the

interaction between the TGF-β1 Smad signaling pathway and intracellular Ca^{2+} signaling pathway.

The role of the Ca^{2+} signal in HOB adhesion enhancement by TGF-β1 is not known. However, increased collagen transcription and integrin subunit stability, two factors which were previously shown to contribute to osteoblast adhesion [Sinha et al., 1994; Frisch et al., 1996], have been suggested as downstream targets of Ca^{2+} signals [Alevizopoulos et al., 1997; Bouvard and Block, 1998]. Thus, a TGF-β1 induced Ca^{2+} response in NIH3T3 fibroblasts has been identified and has been shown to increase the activity of CTF-1, a transcription factor that activates collagen type I expression. Collagen type I comprises more than 90% of the ECM produced by osteoblasts, and we have recently shown that it plays a rate-limiting role in HOB adhesion to orthopaedic implant biomaterials (unpublished data). In addition, we have shown that $\alpha_2\beta_1$, $\alpha_v\beta_1$, and $\alpha_5\beta_1$ integrin subunit expression and distribution are important for osteoblast adhesion to collagen, vitronectin, and fibronectin, respectively, as well as the importance of the TGF-β1 induced Ca^{2+} signal in α5 integrin subunit expression and distribution [Shah et al., 1999; Nesti et al., 2002]. These integrin subunits bind to ECM components such as collagen, vitronectin, and fibronectin to effectively "anchor" the osteoblast to its substratum. Recent studies have shown that $\beta_5\beta_1$ integrin binding to fibronectin is dependent on calcium/calmodulin-dependent protein kinase II (CaMKII) [Bouvard and Block, 1998]. This presents another candidate downstream target for the TGF-β1 induced intracellular Ca^{2+} signal that may contribute to the adhesion enhancement properties of TGF-β1. Of particular interest is our finding of the $[Ca^{2+}]_i$ oscillatory response to TGF-β1, that may act as a maintenance signal and as a contributing mechanism to cell adhesion to substrate via inside-out signaling. These areas merit additional investigation.

It is noteworthy that TGF-β1 utilizes more than one signaling pathway to elicit cell responses. The literature of TGF-β1 action on osteoblasts is replete with apparent inconsistencies. In some instances, TGF-β1 increases osteoblast specific proteins and gene expression [Pfeilschifter et al., 1987; Hock et al., 1990; Bonewald et al., 1992; Mundy et al., 1993], whereas in other cases TGF-β1 may suppress osteoblast function [Noda and Rodan, 1986; Centrella et al., 1987; Chen et al., 1991; Harris et al., 1994]. These differences have often been attributed to the difference between various osteoblastic cell lines and their varying stages of differentiation.

Several authors have shown that the expression levels of Ca^{2+} channels vary in osteoblastic cell lines [Meszaros et al., 1996; Barry, 2000]. In addition, Ca^{2+} channel expression and activity change with osteoblast differentiation [Loza et al., 1994; Preston et al., 1996]. Taken together with the findings reported here, these data may explain, at least in part, the variations in TGF-β1 responses seen in osteoblastic cells. Since the TGF-β1 induced Ca^{2+} signal is initiated by Ca^{2+} influx through L-type channels, the activity of this branch of the TGF-β1 signaling pathway would depend on the number of channels expressed or other regulators of channel activity. These multiple signaling pathways initiated by TGF-β1 may thus contribute to the complex effects of the growth factor reported.

In assessing the in vivo significance of the in vitro findings reported here, two issues should be considered: (1) TGF-β1 concentrations, and (2) use of polystyrene as a culture substrate. While the concentration of TGF-β1 in the osteoblast microenvironment is not known, it is likely that osteoblasts are exposed to a wide range of TGF-β1 concentrations. For example, under conditions of rapid bone turnover or repair, the osteoblast may experience high levels of TGF-β1, whereas in relatively quiescent conditions, the osteoblast may encounter low concentrations of TGF-β1. The concentration of 10 ng/ml of TGF-β1 is found to be within this concentration range [Wildemann et al., 2004]. Although osteoblast adhesion to plastic does not occur in vivo, the events and mechanisms involved in the adhesion process are likely to be similar, such that the in vitro system should serve as a useful experimental model.

In summary, our findings strongly suggest that intracellular Ca^{2+} signaling is likely to be an important component of the TGF-β1 signal transduction pathway in osteoblasts. We have demonstrated a TGF-β1 induced Ca^{2+} signal in HOB, and have shown the importance of this Ca^{2+} signal in cell adhesion to the substrate. It is noteworthy that intracellular Ca^{2+} signals have been observed as part of the response of osteoblasts to a variety of different osteoactive stimuli. For example, mechanical stimulation,

electrical stimulation, and exposure to various growth factors, hormones, cytokines all generate intracellular Ca^{2+} responses in osteoblasts (see review in Huang and Huang [2005]). Understanding the mechanisms regulating and transducing this intracellular Ca^{2+} signal in the activation of osteoblasts should yield significant insights on bone cell function and help to identify functional targets for the development of novel therapies for clinical orthopedics.

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REFERENCES

Advincula MC, Rahemtulla FG, Advincula RC, Ada ET, Lemons JE, Bellis SL. 2006. Osteoblast adhesion and matrix mineralization on sol-gel-derived titanium oxide. Biomaterials 27:2201–2212.

Alevizopoulos A, Mermod N. 1997. Transforming growth factor-beta: The breaking open of a black box. Bioessays 19:581–591.

Alevizopoulos A, Dusserre Y, Ruegg U, Mermod N. 1997. Regulation of the transforming growth factor beta-responsive transcription factor CTF-1 by calcineurin and calcium/calmodulin-dependent protein kinase IV. J Biol Chem 272:23597–23605.

Barry EL. 2000. Expression of mRNAs for the alpha 1 subunit of voltage-gated calcium channels in human osteoblast-like cell lines and in normal human osteoblasts. Calcif Tissue Int 66:145–150.

Barry EL, Gesek FA, Froehner SC, Friedman PA. 1995. Multiple calcium channel transcripts in rat osteosarcoma cells: Selective activation of alpha 1D isoform by parathyroid hormone. Proc Natl Acad Sci USA 92:10914–10918.

Blobe GC, Schiemann WP, Lodish HF. 2000. Role of transforming growth factor beta in human disease. N Engl J Med 342:1350–1358.

Bonewald LF, Kester MB, Schwartz Z, Swain LD, Khare A, Johnson TL, Leach RJ, Boyan BD. 1992. Effects of combining transforming growth factor beta and 1,25-dihydroxyvitamin D3 on differentiation of a human osteosarcoma (MG-63). J Biol Chem 267:8943–8949.

Bouvard D, Block MR. 1998. Calcium/calmodulin-dependent protein kinase II controls integrin alpha5beta1-mediated cell adhesion through the integrin cytoplasmic domain associated protein-1alpha. Biochem Biophys Res Commun 252:46–50.

Branemark PI, Hansson BO, Adell R, Breine U, Lindstrom J, Hallen O, Ohman A. 1977. Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. Scand J Plast Reconstr Surg Suppl 16:1–132.

Centrella M, McCarthy TL, Canalis E. 1987. Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. J Biol Chem 262:2869–2874.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL. 1994. Transforming growth factor-beta gene family members and bone. Endocr Rev 15:27–39.

Chen TL, Bates RL, Dudley A, Hammonds RG, Jr., Amento EP. 1991. Bone morphogenetic protein-2b stimulation of growth and osteogenic phenotypes in rat osteoblast-like cells: Comparison with TGF-beta 1. J Bone Miner Res 6:1387–1393.

Dallas SL, Sivakumar P, Jones CJ, Chen Q, Peters DM, Mosher DF, Humphries MJ, Kielty CM. 2005. Fibronectin regulates latent transforming growth factor-beta (TGF beta) by controlling matrix assembly of latent TGF beta-binding protein-1. J Biol Chem 280:18871–18880.

Dean DC, Newby RF, Bourgeois S. 1988. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor beta, and cAMP in human cell lines. J Cell Biol 106:2159–2170.

Duncan RL, Akanbi KA, Farach-Carson MC. 1998. Calcium signals and calcium channels in osteoblastic cells. Semin Nephrol 18:178–190.

El-Amin SF, Botchwey E, Tuli R, Kofron MD, Mesfin A, Sethuraman S, Tuan RS, Laurencin CT. 2006. Human osteoblast cells: Isolation, characterization, and growth on polymers for musculoskeletal tissue engineering. J Biomed Mater Res A 76:439–449.

Faler BJ, Macsata RA, Plummer D, Mishra L, Sidawy AN. 2006. Focus on basic science: Transforming growth factor-beta and wound healing. Perspect Vasc Surg Endovasc Ther 18:55–62.

Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol 134:793–799.

Harbers GM, Healy KE. 2005. The effect of ligand type and density on osteoblast adhesion, proliferation, and matrix mineralization. J Biomed Mater Res A 75:855–869.

Harris SE, Bonewald LF, Harris MA, Sabatini M, Dallas S, Feng JQ, Ghosh-Choudhury N, Wozney J, Mundy GR. 1994. Effects of transforming growth factor beta on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblasts. J Bone Miner Res 9:855–863.

Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J. 1989. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J Biol Chem 264:380–388.

Heldin CH, Miyazono K, ten Dijke P. 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465–471.

Hill CS. 1996. Signalling to the nucleus by members of the transforming growth factor-beta (TGF-beta) superfamily. Cell Signal 8:533–544.

Hill CS. 2006. Turning off Smads: Identification of a Smad phosphatase. Dev Cell 10:412–413.

Hock JM, Canalis E, Centrella M. 1990. Transforming growth factor-beta stimulates bone matrix apposition and bone cell replication in cultured fetal rat calvariae. Endocrinology 126:421–426.

Huang SS, Huang JS. 2005. TGF-beta control of cell proliferation. J Cell Biochem 96:447–462.

Ignotz RA, Massague J. 1987. Cell adhesion protein receptors as targets for transforming growth factor-beta action. Cell 51:189–197.

Ishiyama N, Shibata H, Kanzaki M, Shiozaki S, Miyazaki J, Kobayashi I, Kojima I. 1996. Calcium as a second messenger of the action of transforming growth factor-beta on insulin secretion. Mol Cell Endocrinol 117:1–6.

Javelaud D, Mauviel A. 2005. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: Implications for carcinogenesis. Oncogene 24:5742–5750.

Lee JY, Kim KH, Shin SY, Rhyu IC, Lee YM, Park YJ, Chung CP, Lee SJ. 2006. Enhanced bone formation by transforming growth factor-beta1-releasing collagen/chitosan microgranules. J Biomed Mater Res A 76:530–539.

Loza J, Stephan E, Dolce C, Dziak R, Simasko S. 1994. Calcium currents in osteoblastic cells: Dependence upon cellular growth stage. Calcif Tissue Int 55:128–133.

Meszaros JG, Karin NJ, Farach-Carson MC. 1996. Voltage-sensitive calcium channels in osteoblasts: Mediators of plasma membrane signalling events. Connect Tissue Res 35:107–111.

Mundy G, Harris S, Sabatini M, Gutierrez G, Garrett I, Izbicka E. 1993. The use of osteosarcoma cells to characterize factors which regulate bone cells function. In: Novack J, McMaster J, editors. Frontiers of osteosarcoma research. Seattle: Hogrefe and Huber. pp 449–456.

Nesti LJ, Caterson EJ, Wang M, Chang R, Chapovsky F, Hoek JB, Tuan RS. 2002. TGF-beta1 calcium signaling increases alpha5 integrin expression in osteoblasts. J Orthop Res 20:1042–1049.

Noda M, Rodan GA. 1986. Type-beta transforming growth factor inhibits proliferation and expression of alkaline phosphatase in murine osteoblast-like cells. Biochem Biophys Res Commun 140:56–65.

Noth U, Tuli R, Seghatoleslami R, Howard M, Shah A, Hall DJ, Hickok NJ, Tuan RS. 2003. Activation of p38 and Smads mediates BMP-2 effects on human trabecular bone-derived osteoblasts. Exp Cell Res 291:201–211.

Oide H, Thurman RG. 1994. Hepatic Ito cells contain calcium channels: Increases with transforming growth factor-beta 1. Hepatology 20:1009–1014.

Pfeilschifter J, D'Souza SM, Mundy GR. 1987. Effects of transforming growth factor-beta on osteoblastic osteosarcoma cells. Endocrinology 121:212–218.

Preston MR, el Haj AJ, Publicover SJ. 1996. Expression of voltage-operated Ca2+ channels in rat bone marrow stromal cells in vitro. Bone 19:101–106.

Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, Yamada SS, Chen WT, Yamada KM, McDonald JA. 1988. Transforming growth factor beta stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J Biol Chem 263:4586–4592.

Robey PG, Termine JD. 1985. Human bone cells in vitro. Calcif Tissue Int 37:453–460.

Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB. 1987. Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro. J Cell Biol 105:457–463.

Rooney TA, Sass EJ, Thomas AP. 1989. Characterization of cytosolic calcium oscillations induced by phenylephrine and vasopressin in single fura-2-loaded hepatocytes. J Biol Chem 264:17131–17141.

Sastry SK, Horwitz AF. 1996. Adhesion-growth factor interactions during differentiation: An integrated biological response. Dev Biol 180:455–467.

Schmidmaier G, Wildemann B, Ostapowicz D, Kandziora F, Stange R, Haas NP, Raschke M. 2004. Long-term effects of local growth factor (IGF-I and TGF-beta 1) treatment on fracture healing. A safety study for using growth factors. J Orthop Res 22:514–519.

Shah A, Hickok N, Chang R, Sinha R, Tuan R. 1997. TGF-b1 Enhances Osteoadhesive and osteomorphogenetic events of primary human osteoblasts on orthopaedic biomaterials. Mol Biol Cell 8:72a.

Shah AK, Lazatin J, Sinha RK, Lennox T, Hickok NJ, Tuan RS. 1999. Mechanism of BMP-2 stimulated adhesion of osteoblastic cells to titanium alloy. Biol Cell 91:131–142.

Shao Y, Alicknavitch M, Farach-Carson MC. 2005. Expression of voltage sensitive calcium channel (VSCC) L-type Ca(v)1.2 (alpha(1C)) and T-type Ca(v)3.2 (alpha(1H)) subunits during mouse bone development. Dev Dyn 234:54–62.

Sinha RK, Tuan RS. 1996. Regulation of human osteoblast integrin expression by orthopedic implant materials. Bone 18:451–457.

Sinha R, Shah S, Tuan R. 1991. Cell spreading and attachment of human osteoblasts to metallic substrates. J Cell Biol 115:448a.

Sinha RK, Morris F, Shah SA, Tuan RS. 1994. Surface composition of orthopaedic implant metals regulates cell attachment, spreading, and cytoskeletal organization of primary human osteoblasts in vitro. Clin Orthop Rel Res 305:258–272.

Thapa N, Kang KB, Kim IS. 2005. Beta ig-h3 mediates osteoblast adhesion and inhibits differentiation. Bone 36:232–242.

Tsien RY. 1989. Fluorescent indicators of ion concentrations. Methods Cell Biol 30:127–156.

Tsien RW, Hess P, McCleskey EW, Rosenberg RL. 1987. Calcium channels: Mechanisms of selectivity, permeation, and block. Annu Rev Biophys Biophys Chem 16:265–290.

Verschueren K, Huylebroeck D. 1999. Remarkable versatility of Smad proteins in the nucleus of transforming growth factor-beta activated cells. Cytokine Growth Factor Rev 10:187–199.

Wang JL, Lin KL, Chen JS, Lu YC, Jiann BP, Chang HT, Hsu SS, Chen WC, Huang JK, Ho CM, Jan CR. 2004. Effect of celecoxib on Ca2+ movement and cell proliferation in human osteoblasts. Biochem Pharmacol 67:1123–1130.

Wierda WG, Mehr DS, Kim YB. 1989. Comparison of fluorochrome-labeled and 51Cr-labeled targets for natural killer cytotoxicity assay. J Immunol Methods 122:15–24.

Wildemann B, Schmidmaier G, Brenner N, Huning M, Stange R, Haas NP, Raschke M. 2004. Quantification, localization, and expression of IGF-I and TGF-beta1 during growth factor-stimulated fracture healing. Calcif Tissue Int 74:388–397.

Yoshida K, Matsuzaki K, Mori S, Tahashi Y, Yamagata H, Furukawa F, Seki T, Nishizawa M, Fujisawa J, Okazaki K. 2005. Transforming growth factor-beta and platelet-derived growth factor signal via c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation in rat hepatic stellate cells after acute liver injury. Am J Pathol 166:1029–1039.

Zahanich I, Graf EM, Heubach JF, Hempel U, Boxberger S, Ravens U. 2005. Molecular and functional expression of voltage-operated calcium channels during osteogenic differentiation of human mesenchymal stem cells. J Bone Miner Res 20:1637–1646.