Journal of Cellular Biochemistry 103:383–392 (2008)

Estrogen-TGFβ Cross-Talk in Bone and Other Cell Types: Role of TIEG, Runx2, and Other Transcription Factors

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Abstract It is well established that E_2 and TGFβ have major biological effects in multiple tissues, including bone. The signaling pathways through which these two factors elicit their effects are well documented. However, the interaction between these two pathways and the potential consequences of cross-talk between E_2 and TGFβ continue to be elucidated. In this prospectus, we present known and potential roles of TIEG, Runx2, and other transcription factors as important mediators of signaling between these two pathways. J. Cell. Biochem. 103: 383–392, 2008.

Key words: Bone; Runx2; TIEG; Estrogen; TGFβ; osteoblast; osteoclast

Estrogen (E_2) and TGFβ are known to be major regulators of skeletal formation and maintenance [Khosla et al., 1999; Spelsberg et al., 1999; Rickard et al., 2002a; Janssens et al., 2005]. In the past there have been numerous reports about the interaction (cross-talk) between these two important regulators, many of which involve transcription factors. This prospectus summarizes some of these studies and proposes some potential areas of cross-talk in osteoblast (OB) cells which includes Runx2 and another important factor discovered by our laboratory, the TGFβ inducible early gene-1 (TIEG), also known as Krüppel-like transcription factor-10 (KLF-10) [Subramaniam et al., 1995].

Grant sponsor: NIH; Grant numbers: R01 DE-14036, AR52004, AG04875; Grant sponsor: The Breast Cancer Research Foundation.

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Received 20 April 2007; Accepted 24 April 2007

DOI 10.1002/jcb.21425

ESTROGEN ACTION IN OB CELLS

The skeleton is one of the main targets of E_2 action in the body as it regulates bone growth and remodeling. Estrogen has major effects on OB and osteoclast (OC) differentiation, including OB proliferation, differentiation, matrix production, and mineralization [Hughes et al., 1996; Robinson and Spelsberg, 1997; Oursler, 1998; Khosla et al., 1999; Rickard et al., 1999, 2002a; Spelsberg et al., 1999]. Decreased E_2 levels are known to be one of the main causes of osteoporosis [Melton, 1995; Gallagher, 1996] and there is abundant evidence demonstrating a critical role for E_2 in regulating bone metabolism and homeostasis in both men and women [Riggs et al., 2002]. It is important to understand E_2 action in the skeleton to better define the mechanisms of bone loss and in order to develop new approaches to prevent and treat osteoporosis. The primary mechanism by which E_2 elicits its effects on target tissues is by binding to, and activating, the two major estrogen receptor (ER) isoforms, ERα and ERβ. It was originally believed that ERβ only modulated the activity of ERα; however, recent studies by our laboratory and others have demonstrated that these two receptors regulate distinct sets of genes in OBs [Waters et al., 2001; Rickard et al., 2002b; Monroe et al., 2003a;

Kian Tee et al., 2004; Stossi et al., 2004; Monroe et al., 2005]. Adding to this complexity is the identification of a host of nuclear receptor co-activators and co-repressors that modulate E_2 action [McKenna et al., 1999]. OBs express both ERα and ERβ, and both are differentially expressed during OB maturation. The ERα concentrations in rat OB cells increase by almost 10-fold during the differentiation of OB precursor cells into mature OBs, whereas ERβ concentrations increase only slightly, but remain high at all stages [Onoe et al., 1997]. ERβ is the primary ER found in cancellous bone while ERα is more highly expressed in cortical bone [Onoe et al., 1997; Bord et al., 2001b].

Studies utilizing mice, deficient for ERα, ERβ, or both, have yielded significant insight into the role of these receptors in the skeleton [Vidal et al., 2000; Sims et al., 2002, 2003]. Interestingly, deletion of ERα in mice leads to a decrease in bone turnover and an increase in cancellous bone volume in both male and female animals. However, cortical thickness and bone mineral density were reduced in these mice [Sims et al., 2002]. Deletion of ERβ in mice leads to slightly increased trabecular bone volume in females with no changes in the bones of male animals [Sims et al., 2002]. Deletion of both receptors results in significant defects in cortical bone and bone mineral density in both male and female animals, as well as a profound decrease in trabecular bone volume only in female mice with no difference in trabecular bone occurring in males [Sims et al., 2002]. Collectively, these data demonstrate that ERβ plays an important role in bone remodeling only in female animals, while ERα is involved in this process in both sexes.

TGFβ Action in OB Cells

TGFβ is produced by OBs and OCs, is localized in large quantities in the skeleton and plays a major role in OB and OC functions [Sanford et al., 1997; Geiser et al., 1998; Filvaroff et al., 1999; Spelsberg et al., 1999; Janssens et al., 2005]. The TGFβ family is composed of three highly related isoforms, TGFβ1, β2, and β3 transcribed from different genes on different chromosomes. TGFβ1 is present in the greatest abundance in bone [Seyedin et al., 1985]. This family of growth factors is known to be induced by estrogen in multiple cell types including human OBs [Oursler et al., 1991a] and OCs [Oursler et al., 1994]. In spite of their high sequence homology, the in vivo functions of the three TGFβ isoforms are highly divergent as analyzed by gene knockouts. Despite conflicting results, the majority of studies indicate that TGFβ1 increases bone formation by recruiting OB progenitors and stimulating their proliferation, resulting in an increased number of cells committed to the OB lineage. Additionally, TGFβ promotes early stages of OB differentiation (bone matrix production), while it blocks later stages of differentiation and mineralization [Alliston et al., 2001; Maeda et al., 2004].

Mice, deficient for TGFβ1, β2, or β3, have been shown to develop severe bone defects [Janssens et al., 2005]. Specifically, TGFβ1 null mice display decreased bone mineral content, with a near absence of OBs in trabecular bone resulting in an osteopenic phenotype [Geiser et al., 1998]. TGFβ2 null mice also contain numerous bone defects (bone loss) in the rib, sternum, vertebrae, and long bones [Sanford et al., 1997]. Interestingly, overexpression of TGFβ2 under the control of the osteocalcin promoter also results in an age-dependent loss of bone mass resembling osteoporosis [Erlebacher and Derynck, 1996]. These results are likely explained by the fact that osteocalcin is expressed at late stages of differentiation and the fact that TGFβ inhibits OB differentiation/activity at later stages. Finally, TGFβ3 null mice also exhibit loss of bone [Kaartinen et al., 1995; Proetzel et al., 1995]. In addition to TGFβ KO mice, the disruption of TGFβ signaling has been shown to have a significant impact on bone. Osteoblast-specific overexpression of a truncated TGFβ type II receptor, that is incapable of mediating TGFβ signaling in mice results in an age-dependent increase in trabecular bone mass [Filvaroff et al., 1999]. Since TGFβ is known to inhibit late stage OB differentiation, it is expected that disruption of TGFβ signaling at this time point would result in increased bone formation as is supported by the studies described above. In accordance with the results of the above studies, the disruption of TGFβ signaling through the deletion of Smad 3 results in the expected osteopenic phenotype [Balooch et al., 2005].

Cross-Talk Between the E_2 and TGFβ Signaling Pathways

There are a significant number of reports demonstrating the interactions/cross-talk

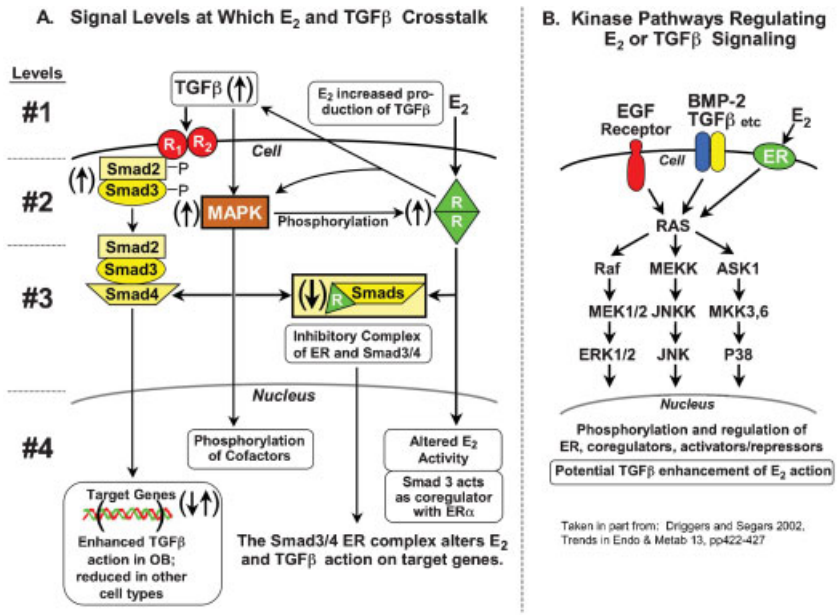

Fig. 1. Outlines of the cellular levels at which estrogen and TGFβ may cross-talk. **A**: Total cellular model identifying the junctures of E_2-TGFβ cross-talk. **B**: Kinase pathways regulating E_2 and TGFβ signaling pathways.

between estrogens and TGFβ signaling pathways in bone [Oursler et al., 1991b; Hughes et al., 1996; Oursler, 1998; Gao et al., 2004; Janssens et al., 2005]. Cross-talk between E_2 and TGFβ occurs at several levels, as depicted in Figure 1A and as described in detail below.

Level 1 Cross-Talk: E_2 Induction of TGFβ in OB and Other Cell Types

There are numerous reports in non-bone cells, as well as OB cells, demonstrating that one level of cross-talk between E_2 and TGFβ involves the E_2 enhancement of the TGFβ pathway via induction of TGFβ gene expression (Fig. 1). This increase in TGFβ production then acts on the surrounding OB cells to activate the TGFβ signaling pathway. The E_2 activation of the TGFβ signaling pathway via induction of TGFβ gene expression has been shown in numerous tissues, including trophoblasts [Rama et al., 2004], bone marrow and prostatic stromal cells [Hong et al., 2004], mouse T cells [Gao et al., 2004], mouse mesenchymal cells [Eger et al., 2004], and mouse adipocytes [Okazaki et al., 2002].

In bone, E_2 induces TGFβ synthesis in several cell types including human OB cells [Oursler et al., 1991a; Hering et al., 1995; Slater et al., 1995; Hughes, 1998; Bord et al., 2001a], human bone marrow cells [Hong et al., 2004], as well as mouse and rat OB cells [Finkelman et al., 1992; Hughes et al., 1996; Narayana Murthy et al., 2006], mouse osteocytes [Heino et al., 2002], and even chicken OCs [Robinson et al., 1996]. In many of the above studies, the levels of TGFβ gene expression or protein were measured, while in other studies TGFβ activity was determined via activation of the R-Smads.

Level 2 Cross-Talk: The MAPK Pathway Amplifies Both E_2 and TGFβ Signaling

A second level of E_2/TGFβ cross-talk, as depicted in Figure 1A,B, involves the TGFβ induced phosphorylation and activation of the ERs and their co-regulators primarily via the MAPK pathway. As depicted in Figure 1B; the E_2-induced MAPK pathway would enhance the TGFβ-MAPK signaling pathway. These pathways are described in reviews [Driggers and Segars, 2002; Gilad et al., 2005]. The activation of the MAPK signaling pathway, as a result of both E_2 and TGFβ, should serve to amplify each others signaling. However, since MAPK activation has been reported to generate an inhibitory phosphorylation of Smad 3 [Javelaud and Mauviel, 2005], E_2 stimulation of MAPK activity via non-genomic pathways with membrane receptors [Gilad et al., 2005], could also result in E_2 inhibition of TGFβ signaling.

Levels 3 and 4 Cross-Talk: The ER-Smad Interaction and Altered Activities by E_2 and TGFβ Signaling Pathway Members

Another level of "cross-talk" between E_2 and TGFβ is the actual interactions of the signaling pathway members with each other (depicted as Level 3 of Fig. 1A) [Paez-Pereda et al., 2005; Andersson and Eggen, 2006]. Since these signaling pathway members are transcription factors, some of these interactions could also be classified as Level 4 (described below). As an example, early studies reported that E_2 reversed the TGFβ induction of type IV collagen promoter activity in a dose-dependent manner in mouse mesangial cells [Silbiger et al., 1998]. Further, gel shift assays, utilizing nuclear extracts and Sp-1 elements in the promoter of type IV collagen, revealed that a TGFβ induced factor was reduced when the cells were treated with E_2. Other studies using human kidney carcinoma cells demonstrated that it is the receptor Smads (−3, −4), interacting with ER, which results in the inhibition of the TGFβ signaling pathway [Matsuda et al., 2001]. This is depicted in Figure 1A. Further studies showed that E_2 blocked Smad 3 activation in both MCF-7 and renal mesangial cells [Malek et al., 2006]. However, estrogen treatment of OBs actually enhances Smad-dependent gene expression in response to TGFβ [McCarthy et al., 2003]. In contrast, TGFβ treatment (i.e., Smad activation) is known to enhance the E_2-induced ERE reporter gene activity in multiple cell types [Malek et al., 2006]. Other studies implicate a role for Smad 4-ERα interactions [Wu et al., 2003; Li et al., 2005]. Smad 4 forms a complex with ERα when the ER binds its target gene promoters to co-repress gene expression. In this latter report, the TGFβ signaling pathway inhibits E_2 target gene transcription via Smad 4-ER interaction.

Level 4. The Cross-Talk Between E_2 and TGFβ at the Level of Transcription Factors and Specific Gene Regulation

Level 4 mechanisms for the inactivation of the TGFβ/Smad pathway have been reported. For example, Ski and Sno oncoproteins inhibit TGFβ signaling by binding Smads 2/3 which together recruit the co-repressor NCOR1 to the Smad binding elements in target genes [Akiyoshi et al., 1999; Stroschein et al., 1999]. This level of cross-talk could also include the Runx2 and TIEG proteins, which are also transcription factors. The level 4 cross-talk described below focuses mainly on the role of TIEG and Runx2 in the E_2-TGFβ cross-talk pathways (see Figs. 1 and 3).

POTENTIAL ROLES OF TIEG AND RUNX2 IN THE E_2/TGFB CROSS-TALK

Biological Functions of TIEG

Since the discovery of TIEG by this laboratory [Subramaniam et al., 1995], there have been over 70 publications regarding the functions of TIEG, and its immediate family members, in numerous cell types. TIEG has been implicated as a marker for breast cancer [Subramaniam et al., 1998; Reinholz et al., 2004], as playing a role in cell differentiation [Noti et al., 2004; Subramaniam et al., 2005], as a target gene for a variety of signaling pathways [Subramaniam et al., 1995; Hefferan et al., 2000a; Wahab et al., 2005], and finally as displaying abnormal expression in diseased tissues [Subramaniam et al., 1998; Reinholz et al., 2004]. TIEG is expressed in numerous tissues and is a member of the Krüppel-like family of transcription factors (KLF-10) which are known to be involved in antiproliferative and apoptotic inducing functions similar to that of TGFβ [Dang et al., 2000]. KLF family members bind to Sp-1/GC-rich DNA elements via their zinc fingers to regulate gene expression. The TIEG gene encodes a 480 amino acid (72 kDa) protein with a unique amino-terminal end which distinguishes it from an early growth response-α (EGRα) gene produced from an alternate promoter [Blok et al., 1995; Subramaniam et al., 1995; Fautsch et al., 1998b]. Overall, the N-terminal region of TIEG represents the activation domain, the middle region, the repression domain, and the C-terminal region, the DNA binding domain. The C-terminal DNA binding domain of TIEG has more than 90% homology to other Sp-1-like transcription factor family members, including TIEG2 and TIEG3. However, the N-terminal domains are largely non-homologous. TIEG has several SH-3 binding domains in this N-terminal region through which other proteins may interact to aid TIEG in the regulation of target gene expression. TIEG is known to activate as well as repress the transcription of a number of genes [Johnsen et al., 2002a; Noti et al., 2004].

In summary, TIEG has been shown: to be induced by E_2, TGFβ1, 2, 3, EGF, and BMP-2, and to repress the expression of Smad 7, resulting in activation of the TGFβ-Smad signaling pathway [Johnsen et al., 2002a]. TIEG has also been shown to induce the expression and activity of R-Smad 2 [Johnsen et al., 2002b]; and to be rapidly turned over by the E_3-ubiquitin ligase pathway [Johnsen et al., 2002c]. Finally, TIEG has been shown to play a major role in the TGFβ inhibition of cell proliferation [Johnsen et al., 2004]. In fact, overexpression of TIEG in human OB cells mimics the actions of TGFβ [Hefferan et al., 2000a]. The role of TIEG in regulating the TGFβ-Smad signaling pathway is depicted in Figure 2.

In order to better understand the function of TIEG in bone, our laboratory has generated TIEG null ($TIEG^{-/-}$) mice and have found that females, but not males, have smaller and weaker bones, relative to wild-type littermates, which can be characterized as osteopenic [Bensamoun et al., 2006a]. This phenotype is interesting since ERβ, which regulates TIEG expression, has also been shown to play an important role in bone remodeling only in female animals [Sims et al., 2002]. In addition to E_2, deletion or disruption of TGFβ signaling in mice results in an osteopenic phenotype unless the disruption occurs specifically in OB during late stages of differentiation, as discussed previously. These studies are also in agreement with the bone phenotype observed in

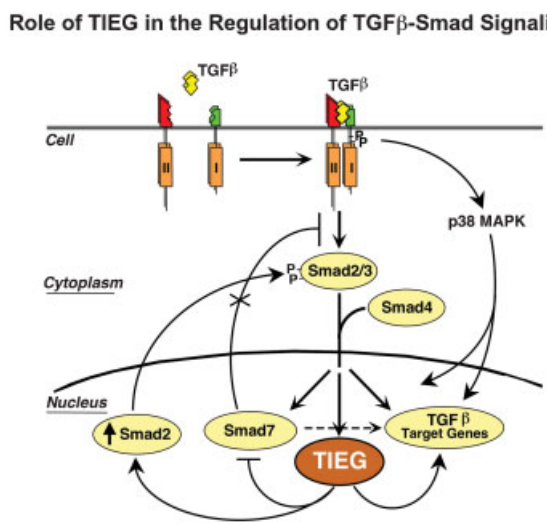

Fig. 2. Mechanisms of action of TIEG in the TGFβ-Smad signaling pathway.

$TIEG^{-/-}$ mice since we have shown that TIEG plays an important role in eliciting the effects of TGFβ signaling in OBs. Our laboratory has reported that calvarial OBs isolated from $TIEG^{-/-}$ mice have a markedly reduced capacity to mineralize bone and to support OC differentiation [Subramaniam et al., 2005]. Further characterization of these OBs in our laboratories have revealed decreased expression levels of Runx2, osterix, alkaline phosphatase, and other important OB marker genes.

INTERACTIONS OF TIEG AND RUNX2 IN THE E_2/TGFB CROSS-TALK PATHWAYS

E_2/TGFβ Induction of TIEG

Following the discovery of ER expression in human OBs, our laboratory identified TGFβ1 as one of the few growth factors known to be regulated by E_2 in these cells [Oursler et al., 1991a]. In order to further understand the molecular mechanisms of TGFβ action in OBs, gene expression studies were performed which identified TIEG as an early response gene to TGFβ treatment [Subramaniam et al., 1995]. Recently, it was shown that E_2 also induces the expression of TIEG in an ER isoform specific manner (unpublished). As depicted in Figure 3, since E_2 and TGFβ both induce the expression of TIEG, a synergistic interaction between these two ligands is likely to occur.

Role of TIEG in the E_2/TGFβ Induction of Runx2

Recent studies in our laboratory have shown that TIEG directly induces the expression of Runx2 in human and mouse OB cells (unpublished) as well as repress the expression of osteoprotegerin (OPG) [Subramaniam et al., 2005]. This implicates a role for TIEG in osteoblastogenesis as well as osteoclastogenesis and, in part, explains the TIEG knockout mouse skeletal phenotype and the OB defects. Runx2 is a major OB lineage-determining transcription factor involved in directing precursor stem cells to the preosteoblast lineage and their concomitant differentiation [Shinke and Karsenty, 2002; Lian et al., 2004]. Runx2 appears to be the master gene for OB differentiation since Runx2 null mice have only a cartilaginous skeleton [Komori et al., 1997; Otto et al., 1997]. Additionally, Runx2 also induces the expression of osterix, a transcription factor which is required to finalize terminal OB

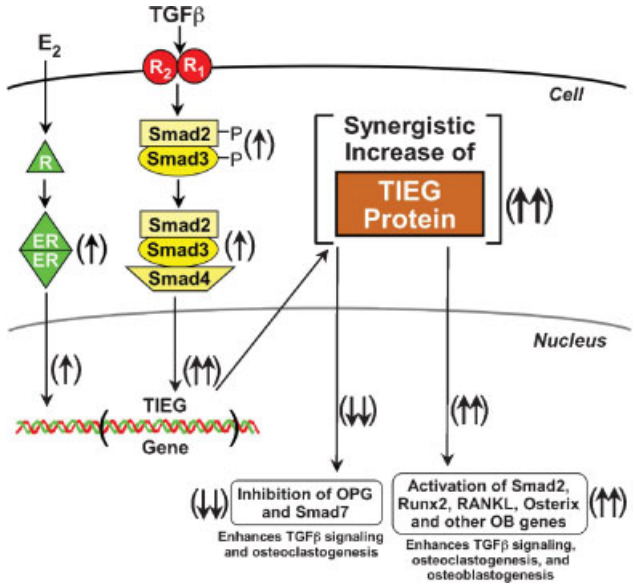

Fig. 3. Potential role of TIEG in the synergistic actions of E_2 and TGFβ pathways.

differentiation [Nakashima et al., 2002]. Osterix-null mice fail to form bone since the OB precursor cells cannot differentiate into mature OBs, even though these cells express Runx2 [Nakashima et al., 2002]. Osterix expression is induced by, and collaborates with, Runx2 to activate OB specific genes related to matrix production and the mineralization processes [Nakashima et al., 2002].

Past studies have shown that TGFβ and BMP-2 indirectly induces Runx2 expression [Lee et al., 2000, 2002]. However, the mediator of this action is unknown. Similar studies have also reported that Runx2 can mediate the actions of E_2 in a tissue specific manner [Sasaki-Iwaoka et al., 1999]. Others have shown that SERMs increase Runx2 promoter activity in U_2OS cells through an AP-1 site adjacent to a Runx2 binding site (OSE) [Tou et al., 2001]. One subsequent report did indicate that E_2 increases Runx2 activity, but not Runx2 transcription, in rat osteoblast [McCarthy et al., 2003]. In these studies, androgens had no effect on Runx2 activity, whereas glucocorticoids inhibited Runx2 activity. Recent studies from our laboratory have documented the increased expression of the endogenous Runx2 gene in primary OB cultures from wild-type mice following E_2 treatment (unpublished). Since this induction of Runx2 by both TGFβ and E_2 is not observed in OBs in which TIEG is absent, a role for TIEG in mediating the TGFβ and E_2 induction of Runx2 expression is suggested (unpublished).

In addition to the E_2 and TGFβ induction of Runx2 gene expression, there are reports of cross-talk between ERα and Runx2 via ERα-Runx2 heterodimer complexes [McCarthy et al., 2003], as well as Smad 2/3-Runx2 heterodimer complexes [Zhang et al., 2000]. Our laboratory has recently found that TIEG protein also interacts with the Runx2 protein (unpublished). The interaction of TIEG and Runx2 could create a tight interactive cross-talk between the E_2 and TGFβ signaling pathways, since both factors (TGFβ and E_2) regulate both TIEG and Runx2 expression. Figure 4 summarizes the reported cross-talking junctures, including new possible

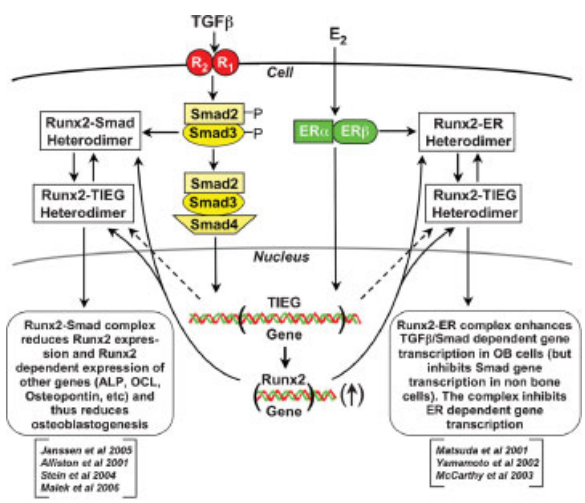

Fig. 4. Potential roles of Runx2 and TIEG in the E_2/TGFβ cross-talk.

cross-talk mechanisms, between E_2 and TGFβ which occur/may occur through the Smads, ER, TIEG, and Runx2. Since E_2 and TGFβ induce TIEG expression, and TIEG has recently been shown not only to regulate Runx2 expression, but also to interact with Runx2 at target gene promoters, and since TIEG seems to be required for the E_2 or TGFβ regulation of Runx2, we hypothesize that TIEG is positioned before Runx2 in the E_2/TGFβ signaling pathways (Fig. 4). Since Runx2 has been shown to interact not only with TIEG, but also with Smads 2/3 [Zhang et al., 2000] and ER [Matsuda et al., 2001; McCarthy et al., 2003], we have included the TIEG-Runx2, TIEG-ER, and TIEG-Smad heterodimers in this figure. The Smad-Runx2 complex reduces Runx2 activity in OBs [Janssens et al., 2005; Malek et al., 2006] while the ER-Runx2 complex reportedly enhances the TGFβ/Smad signaling pathway in these cells [Matsuda et al., 2001; McCarthy et al., 2003]. The exact action of the TIEG-Runx2 heterodimers is currently under study.

SUMMARY

In summary, numerous studies have implicated an important role for E_2 and TGFβ in maintaining normal bone turnover, remodeling, and function. Cross-talk between the two effectors has been documented. TIEG is an immediate response gene following E_2 and TGFβ treatment of OBs and is required for OB differentiation and appears to be required for the E_2 and TGFβ regulation of Runx2 gene expression, implying a potentially important role for TIEG in mediating the affects of, and cross-talk between, E_2 and TGFβ in target cells. As has been demonstrated throughout this prospectus, this cross-talk includes the E_2 induction of TGFβ gene expression which results in activation of the Smad signaling pathway. In addition, E_2 and TGFβ are also known to activate the kinase pathways which can, in turn, activate nuclear co-regulators, Smads, and ER itself. Finally, interactions between ERs, the Smad signaling components, TIEG, Runx2, and TGFβ have now been documented, rendering multiple cross-talk junctures between the E_2 and TGFβ signaling pathways. Overall, an important role for TIEG in mediating both E_2 and TGFβ signaling, as well as cross-talk between these two important pathways, has been revealed.

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