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Characterization of Estrogen Receptor α in Mouse Osteoblasts

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Titel: Characterization of Estrogen Receptor alpha in Mouse Osteoblasts

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Zusammenfassung

Östrogene sind für die Koordinierung der reproduktiven Organe verantwortlich. Zusätzlich beeinflussen sie nichtreproduktive Organe wie Knochen durch die Regulierung von Osteoblasten und Osteoklasten, die die Knochenhomöostasis kontrollieren. Östrogene können zwei nukleäre Rezeptoren aktivieren, Östrogen Rezeptor (ER) alpha und ER beta. Nach Östrogenbindung aktivieren ERs direkt die Transkription von Zielgenen, welche das Zellschicksal bestimmen. Osteoblasten exprimieren ER alpha und wurden als direktes Target und Haupteffektor von Östrogen vorgeschlagen.

Um die Osteoblastenregulation besser verstehen zu können, wurden in dieser Studie die Funktion von ER alpha in primär kultivierten Mausosteoblasten und mesenchymalen Zellinien charakterisiert. Unsere Ergebnisse bestätigen, dass ER alpha in Osteoblasten exprimiert wird und dass transkriptionelle Aktivierung durch ER alpha stattfinden kann. Allerdings waren die Expressionslevels sehr viel niedriger als in reproduktiven Geweben. Zu unserer Überraschung hatte ER alpha in Osteoblasten eine hohe transkriptionelle Aktivität, was allerdings von niedrigen Konzentrationen von Östrogen im Wachstumsmedium abhing. ER alpha Expression erhöht sich wärend der Zelldifferenzierung. In früheren Studien wurde vermutet, dass dies die Spezifität von ER alpha Expression in Osteoblasten widerspiegelt. Unsere Ergebnisse zeigen allerdings, dass ER alpha Expression auch in anderen Zelltypen wärend der Differenzierung induziert wird. Ausserdem konnte kein Einfluss von Östrogen auf die Osteoblastendifferenzierung festgestellt werden. Zusammengefasst bestätigt diese Studie, dass funktionelle ER in Osteoblasten exprimiert werden. Allerdings stellen unsere Ergebnisse die Hypothese in Frage, dass Osteoblasten als direkte Mediatoren für Östrogen in der Regulation der Knochenhomöostasis agieren.

Summary

Estrogens are commonly known for coordinating reproductive function. However, they also affect the physiology of non-reproductive tissues such as bone. In particular, they regulate two cell types that control bone homeostasis, osteoblasts and osteoclasts. Estrogens can activate two nuclear receptors, Estrogen Receptor (ER) alpha and ER beta. After binding to estrogen, ERs directly activate transcription target genes that determine cell fate. osteoblasts express ER alpha and have been suggested as the direct target of estrogens that mediates most effects estrogens have on bone.

To better understand Osteoblast regulation by estrogen, we characterized ER alpha in primary mouse osteoblasts and mesenchymal cell lines. This study confirms that ER alpha is expressed in osteoblasts and that it can mediate transcriptional activation. However, the expression level of ER alpha is very low as compared to reproductive tissues. Surprisingly, ER alpha in osteoblasts still has a high residual transcriptional activity, which depends on low concentration of estrogens in the growth medium.

ER alpha expression increases during differentiation of mouse osteoblasts. Previous studies suggested that this might reflect the specificity of ER alpha expression in osteoblasts. However, our results demonstrate that induction of ER alpha expression upon differentiation is not limited to osteoblasts as differentiation of a mesenchymal cell line into myoblasts also increases the levels of ER alpha expression. Finally, Estrogens did not influence osteoblast differentiation. In conclusion, these results confirm the presence of a functional ER in osteoblasts, but challenge the view that osteoblasts act as direct mediators of estrogen action on bone homeostasis.

Introduction

1. Introduction: a historical overview

It was in the 1920's that a hormone present in the ovaries, able to induce estrus or ovulation as well as a swelling of the uterus and the vagina, was isolated (Allen and doisy, 1923). This hormone was, as a result of its effects, termed estrogen. The principal physiological form of the hormone is 17-β-Estradiol (E2). It remained however unknown how estrogen could achieve its effects on the reproductive tract until the early 1960's, when the use of radiolabeled E2 with high specific activity allowed the localization of an estrogen binding protein or estrogen receptor (ER) to target tissues mostly the uterus vagina and the anterior pituitary (Jensen and Jacobson, 1962). Estrogens were later found to alter RNA polymerase II (RNA pol II) transcription in target tissues suggesting ER was a transcription factor (O'Malley and McGuire, 1968). Following progress in molecular biology techniques, the cDNA of the first ER was then cloned (Green et al., 1986;Greene et al., 1986). The cloning of ER followed that of other intracellular hormone receptors, namely the glucocorticoid receptor (Weinberger et al., 1985) and the thyroid receptor (Sap et al., 1986;Weinberger et al., 1986) which all shared homologous sequences. Collectively, sequence comparisons determined the existence of a family of ligand-regulated transcription factors that shared a conserved sequence and apparently modular organization (Evans, 1988). Nuclear receptors, including ER, become transcriptionally active upon ligand binding. They generally bind to DNA as dimers, thereby recognizing a cognate palindromic DNA sequence, and thereafter recruit the transcriptional machinery to a target promoter (Kumar et al., 1987).

In the late 80's, in a process termed "squelching", it was found that transcription factors could compete for limiting factors of the intermediate transcription machinery,

thereby inhibiting the transcriptional capacity of each other (Meyer et al., 1989). These shared factors required for nuclear receptor transcriptional activation were termed coactivators. The 90's were dedicated to the identification of these coactivators. The first coactivators were isolated in 1994 (Halachmi et al., 1994;Cavailles et al., 1994). Their cloning was achieved a year later (Cavailles et al., 1995;Onate et al., 1995). A multitude of coactivators was subsequently cloned (McKenna et al., 1999). It was realized that some coactivators had enzymatic activity and could covalently modify histones or remodel chromatin around the binding site of ER (Fryer and Archer, 1998;Blanco et al., 1998). Other coactivators forming the mediator complex and involved in promoting the activation of polII on the promoter, do not contain any enzymatic activity but directly contact and recruit the transcriptional machinery to the remodeled chromatin (Malik and Roeder, 2000). Corepressors were also concomitantly identified and showed opposite enzymatic activity to that of coactivators thus opposing their effects (Heinzel et al., 1997;Nagy et al., 1997).

The development of chromatin immunoprecipitation (ChIP) in mammalian cells allowed the characterization of the binding kinetics of the different factors to the promoter of a target gene and revealed the dynamic nature of transcription activation (Shang et al., 2000;Metivier et al., 2003). The use of GFP tagged nuclear receptors confirmed the dynamism of the interaction between nuclear receptors and a promoter (McNally et al., 2000).

Cloning of a second, genetically distinct ER (coined ER β, ER α corresponding to the first ER cloned), raised the question as to which one of the two ERs mediated which effects of estrogens (Kuiper et al., 1996). The use of gene-targeting technology confirmed that most of the effects of estrogens on the reproductive tract are mediated

by ER α with ER β playing a mostly redundant function (Dupont et al., 2000). Additionally, knockout mice devoid of either ER α, ER β or both confirmed that the physiological effects of estrogens as anticipated, are mediated by estrogen receptors with ER α having the major physiological influence.

2. Structural and functional aspects of ER α

Estrogen receptor α belongs to the super-family of nuclear receptors. Nuclear receptors share a common modular and functional organization. This was revealed by multiple sequence alignment of the predicted primary sequence of nuclear receptors, where six different regions with different extent of sequence conservation were delineated. These regions correspond approximately to different functional domains of the receptor (Krust et al., 1986;Evans, 1988). These domains were defined as A to F and this terminology is used here. The numbering of residues corresponds, unless otherwise mentioned, to the human ER α protein. The location of the different domains and functions of ER α are depicted in figure 1.

It has not, to date, been possible to determine the tertiary structure of the whole ER α or for that matter of any other nuclear receptor. As a result, direct structural evidence is not available on the interplay between different domains. Although these domains display a certain functional autonomy it was realized that the steroid receptor protein functioned as a whole and that different domains could interact either directly or allosterically to influence each other's function. One example of intramolecular interaction will be detailed also be detailed here.

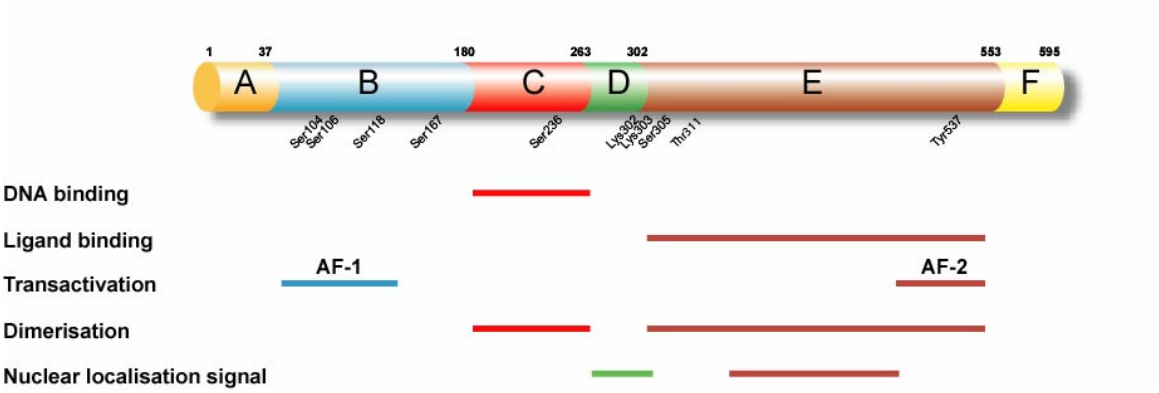


Figure 1. Functional Domains of ER α

The different domains of ER α are represented with their associated functions. Residues targets of posttranslational modifications are indicated below. Numbering corresponds to the position of the domains in the human receptor in amino acids.

2.1. ER α functional domains

Domain A (Amino Acids 1-37)

The A domain is not found in every nuclear receptor and, previously, no function had been assigned to it (Metzger et al., 1995). Very recently however, it was found that the A domain, and more precisely a LLxxI motif within the A domain, interacts with the E domain, and by doing so inhibits, through direct competition, ligand-independent recruitment of coregulatros by the activation functions of ER α (Metivier et al., 2002f). This motif is not found in other nuclear receptors and is not even present in the closely related ER β; this function therefore constitutes a unique feature of ER α (Metivier et al., 2002e). The interaction between the A and E domains will be detailed later in the introduction.

Domain B (Amino Acids 38-180)

This domain is present in most nuclear receptors; it is however very variable both in length and composition (Evans, 1988). The B domain contains the first activation function of nuclear receptors (AF-1). Although the whole receptor is generally inactive in the absence of hormone, the isolated A/B domain can transactivate constitutively when fused to a DNA binding domain (Kumar *et al.*, 1987;Tora et al., 1989b;Berry et al., 1990;Metzger *et al.*, 1995). The AF-1 of ER α and other nuclear receptors is unlike the activation domains of other transcription factors (Tasset et al., 1990). Despite this distinct primary structure, ER α AF-1 functions like other transcription factors, by recruiting coactivators and does so in a ligand independent manner when isolated from the rest of the receptor (Tasset *et al.*, 1990;Webb et al., 1998).

Conflicting data are available as to the precise location of AF-1 within the B domain, but at most it spans the region from residue 38 to 127 (Metzger *et al.*, 1995;McInerney and Katzenellenbogen, 1996;Webb *et al.*, 1998). The activity of AF-1 was also shown to be entirely dependent on the integrity of an evolutionary conserved putative α-helix (amino acids 39-44; Metivier et al., 2000). The structural basis of coactivator binding is also not known and would involve a different type of interaction as to what as been characterized for AF-2. Different discreet binding sites have been mapped on ER α for different categories of coactivators and would encompass residues 38-127 (Webb *et al.*, 1998;Endoh *et al.*, 1999;Kobayashi et al., 2000).

The B domain also contains several residues that can be phosphorylated. Best characterized are serine residues 104, 106, 118 and 167. Phosphorylation of these

residues have been shown to be important for receptor functions ranging from dimerization to transcriptional activation (Lannigan, 2003).

Domain C (Amino Acids 181-263)

The C domain is the most conserved among nuclear receptors (Evans, 1988). This domain contains the DNA-binding domain (DBD; Kumar *et al.*, 1987). ER α, like other class I nuclear receptors, binds to a palindromic DNA sequence, consequent on dimerization (Kumar and Chambon, 1988;Green et al., 1988). The consensus sequence of the estrogen response element (ERE) to which ER α binds is AGAACAnnnTGTTCT, n being any nucleotide constituting the spacer (Klein-Hitpass et al., 1988;Klinge, 2001).

The structure of the DBD is organized around two zinc-binding Cys2-Cys2 sequence motifs, known as zinc fingers. This widespread DNA binding motif is conserved in every nuclear receptor (Freedman et al., 1988;Schwabe et al., 1990). The structure of domain C was the first to be solved for ER α (Schwabe *et al.*, 1990;Schwabe et al., 1993). The 3D structure confirmed the presence of the two zinc fingers and revealed two amphipathic α-helices adjacent C-terminal to them (Schwabe *et al.*, 1993). Each module, consisting of a single zinc finger and one α-helix, is joined with one β-turn. The binding to DNA is achieved through the first α-helix that binds in the major groove of target DNA. The D-box, in the C-terminal part of the second zinc finger, generates the dimerisation interface between two DBD monomers.

The responsive elements within promoters influenced by different nuclear receptors are very similar in sequence but yet specifically associate with their respective receptors. The specificity of binding is achieved by the so-called P-box located in

helix I. Mutation of three residues changes, for instance, the binding characteristics of the ER α DBD into that of the glucocorticoid receptor (Mader et al., 1989).

In terms of posttranslational modification, serine 236 has been shown to be the target of phosphorylation thereby affecting receptor dimerization (Chen et al., 1999).

Domain D (Amino Acids 264-302)

This domain is also called the hinge region and is variable in size and sequence (Krust *et al.*, 1986;Evans, 1988). In terms of function, the domain D harbours most of the constitutive nuclear localization signal, comprised between residues 256 and 303 (Picard et al., 1990;Ylikomi et al., 1992). The C-terminal part of the hinge region in ER α contains part of an ill-characterized activation function called AF-2a (Norris et al., 1997).

Domain E (Amino Acids 303-553)

This domain, although variable in terms of sequence, is structurally very well conserved among nuclear receptors (Wurtz et al., 1996). Domain E corresponds to the ligand-binding domain (LBD; Kumar *et al.*, 1987) and also harbours the ligand-dependent activation function (Kumar *et al.*, 1987;Bocquel et al., 1989). AF-2 interacts with coactivators in the presence of estradiol (McKenna *et al.*, 1999) while in the presence of an antagonist like tamoxifen, it associates with corepressors (Smith et al., 1997;Metivier et al., 2002b;Liu and Bagchi, 2004). In the absence of ligand the AF-2 binds neither coactivators nor corepressors. The binding of coactivators and corepressors to ER α relies on an LxxLL motif (NR box) predicted to form an amphipathic helix, within the interacting protein (Heery et al., 1997;Shiau et al., 1998;Hu and Lazar, 1999). Coactivators and corepressors associate with an

overlapping but distinct surface on the activated nuclear receptor and as a result their binding is exclusive (Nagy et al., 1999).

The crystal structure of ER α LBD has been determined in the presence of E2 and in association with the antagonists tamoxifen and raloxifene (Brzozowski et al., 1997;Shiau *et al.*, 1998). The structure of the unliganded LBD is however not available. Co-crystals of E2 bound ER α LBD and of a short peptide corresponding to an NR box have also been obtained and the structure solved (Shiau *et al.*, 1998). These structural data reveal that the LBD forms a three-layer α helical sandwich. The hydrophobic ligand is found within a cavity that mainly consists of non-polar amino acids. In the agonist bound conformation, helix 12 of the ER α LBD forms a hydrophobic groove into which the NR box α helix, defined by the LxxLL motif, can bind. However, in the antagonist bound conformation, helix 12 is displaced to cover this hydrophobic groove, thereby preventing interaction with the NR box. It is however not clear if the structural data obtained can be extrapolated to the unliganded LBD of ER α, although as with the antagonist bound receptor, unliganded ER α also bind corepressors in the absence of its N-terminal domain (Metivier *et al.*, 2002b).

The E domain also regulates dimerization following ligand binding (Kumar *et al.*, 1988). The crystal structure of E2 liganded ER α revealed that two LBDs arrange themselves in a head to head manner (Brzozowski *et al.*, 1997).

Several residues within the E domain have been shown to be the target of posttranslational modifications. Serine 305 can be phosphorylated, while lysines 302 and 303 can be acetylated. These modifications affect transcriptional activation by the receptor (Wang et al., 2001;Wang et al., 2002;Balasenthil et al., 2004;Michalides et al., 2004). Threonine 311 can be phosphorylated and regulates nuclear export of the receptor (Bai et al., 1997). Finally tyrosine 537 has been shown to be an important

regulator of receptor functioning and has been shown to be phosphorylated (Arnold et al., 1995).

Domain F (549-595)

This domain is very variable, even between the same nuclear receptor within different species, in terms of sequence but is however very well conserved in terms of length (Nichols et al., 1998). The F domain is not required for the transcriptional activity of ER α (Kumar *et al.*, 1987). However, as it could interact with the LBD it may, in certain contexts, modulate the response to ER α antagonists (Montano et al., 1995;Nichols *et al.*, 1998).

2.2. Interaction between N-and C- terminal domains and the basis of ligand dependent activity

Although the structure of the whole receptor has not been solved, functional interactions have however revealed that both physical and allosteric interactions occur within ER α. One example of physical interaction between two distant domains is described here.

As a result of the deletion of the A domain of ER α, the unliganded receptor can recruit coactivators to its AF-1 and corepressors to the unliganded AF-2 (figure 2; Metivier *et al.*, 2002b). The ligand-independent transactivation and transrepression functions of the receptor are inhibited in the whole ER α, because the A domain interacts with the distant E domain. As a result of the folding of the protein and the intramolecular interactions, in the whole receptor, neither AF-1 nor AF-2 are accessible for protein interactions. The interaction between the two domains is

disrupted upon ligand binding, which then allows binding of coactivators to AF-1 and AF-2. On the other hand binding to a partial antagonist like tamoxifen although it disrupts the interaction between the A and E domains, permits binding of corepressors to AF-2 and binding of coactivators to the newly accessible AF-1.

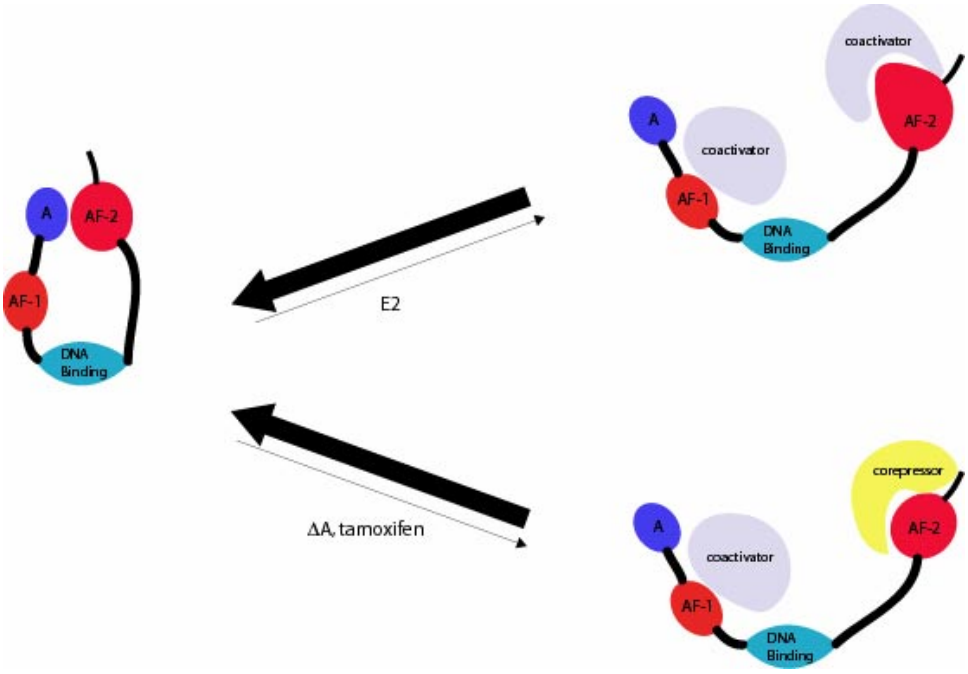


Figure 2. Interaction between the distant A and E domains

In the absence of ligand, the A domain and the AF-2 containing E domain interact with each other. This interaction prevents recruitment of transcription coregulators. The binding of E2 disrupts the interaction and allows the AF-2 and the AF-1 to recruit coactivators. In the presence of the antagonist tamoxifen or after deletion of the A domain, the interaction between the A and E domains does not take place but the AF-2 displays a corepressor binding surface. In this case, the AF-1 can recruit coactivators as long the interaction between the N- and C-terminus is disrupted. Note that a receptor dimer only binds one molecule of coactivator or one of corepressor at a time. As a result depending on the concentrations of corepressor and coactivator in the cell tamoxifen will either recruit coactivators through the AF-1 or corepressors through the AF-2. Two molecules are represented to represent different possibilities.

The A domain directly interacts with the C-terminal region of the receptor; this involves an interaction between an ELE sequence present in the A domain with a

KCK sequence within the E domain (Metivier *et al.*, 2002b). The interaction between the A and E domains permits binding of a LLxxI putative α-helix, present in the A domain of ER α, into the hydrophobic groove of the E domain. The A domain would thereby function as an internal corepressor, competing both with helix 12 corepressors for binding in this cleft. Consequent to the folding of the protein, the AF-1 would not be accessible either to coactivators and would thus be silenced This interaction is predicted to be disrupted upon ligand binding. Consequently, AF-1 and AF-2 can recruit coactivators.

3. ER α mediated transcription

3.1. Mechanism of ER α mediated transcription

When genes are silent, DNA is packaged into a highly organized and compact nucleoprotein structure known as chromatin, which impinge all the transcription steps. The basic unit of the chromatin is the nucleosome, which consists of DNA wrapped twice around an octamer protein core containing two copies each of four histone proteins, H2a, H2b, H3 and H4. Protruding from the nucleosomes are N-terminal histone tails whose interaction with DNA can be modulated upon covalent modifications (Peterson and Laniel, 2004).

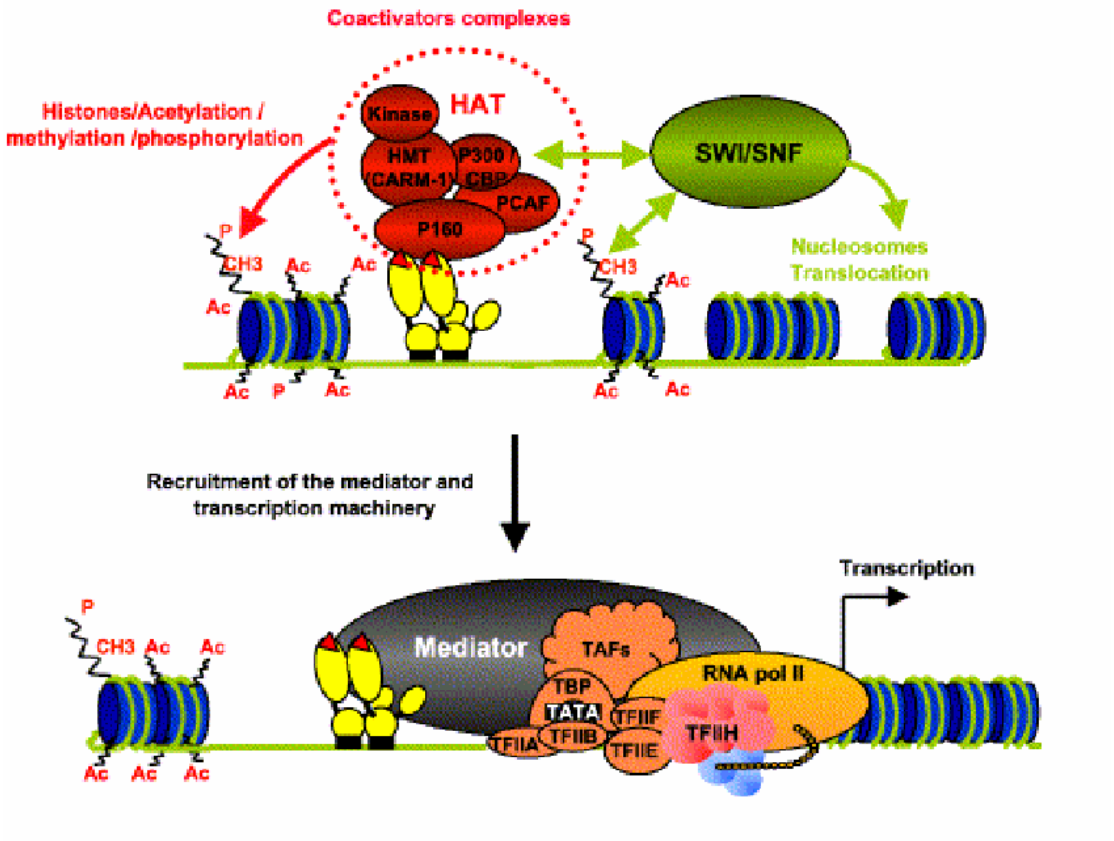


Figure 3. Mechanism of ER α action

Upon ligand binding, coactivators are recruited displaying histone acetyltransferase activity (HAT), methylstransferase, kinase or ATP-dependent remodeling (SWI/SNF) activities, that decompact repressive chromatin. Reruitment of the mediator complex allows entry of the basal transcription machinery which is followed by transcription initiation (adapted from, Bastien and Rochette-Egly, 2004).

To permit transcription, ER α, as with other transcription factors, must initially remodel the chromatin structure surrounding the promoter to allow binding of the transcriptional machinery. To effect chromatin remodeling, the liganded receptor recruits, directly or indirectly, coactivators which covalently modify histones and which induce nucleosome rearrangements (figure 3; Khorasanizadeh, 2004). The covalent modification of histone tails by coactivators is believed to relieve their interactions with the nucleosome DNA, through the acetylation of lysine residues and the methylation of arginine residues. p300/CBP and pCAF possess histone acetyltransferases (HAT) activity that acetylates lysine residues on the histone tails.

CARM-1 acts through its histone methyltransferase activity (HMT) methylating arginine or lysine residues on histone tails. Coactivators of the p160 family like SRC-1, although they display a weak HAT activity, are believed to act as a scaffold protein that recruits other coactivators. ATP-dependent chromatin remodelers (SWI/SNF) are also recruited to ER α activated promoters and reposition nucleosomes at the promoter. The combined action of these coactivators would prepare the chromatin for subsequent binding of the transcription machinery (Narlikar et al., 2002;Metivier *et al.*, 2003).

Once repressive chromatin has been decondensed, the complex induced by activated ER α then recruits the basal transcription machinery. Although ER α can contact members of the RNA pol II complex, it is likely that the productive interaction is indirect, through a protein complex known as the mediator (Malik *et al.*, 2000). Once the transcriptional initiation complex is assembled on the promoter, transcription then initiates.

Kinetically, ChIP analysis performed at short intervals revealed that transcription factors, coregulators and the transcriptional machinery cycle on the promoter in a sequential way (Shang *et al.*, 2000;Hatzis and Talianidis, 2002;Agalioti et al., 2002;Metivier *et al.*, 2003). Modification of local histones was also found to be dynamic and sequential (Metivier *et al.*, 2003). The kinetics of ER α mediated coregulator recruitment was studied in details on the estrogen-responsive pS2 promoter (Metivier *et al.*, 2003). ER α cycles on the pS2 promoter in the absence of ligand confirming that ligand binding is not required for DNA binding (Reid et al., 2003;Metivier et al., 2004). The unliganded receptor however does not recruit the transcriptional machinery to the pS2 promoter. Upon binding E2, cycling of ER α becomes slower. A first transcriptionally unproductive cycle immediately after E2

treatment results in the remodeling of the local nucleosomes and acts to generate a transcriptionally competent conformation. An initial recruitment of the ATP-dependent remodeling factor SWI/SNF occurs, followed by recruitment of HMTs and HATs. Remodeling of chromatin is then followed by recruitment RNA pol II and the mediator complex. Following transcription initiation, ER α and chromatin remodeling complexes are removed from the promoter allowing subsequent cycles to proceed. The histones are then deacetylated by histone deacetylases (HDAC) and remodeled and a new cycle can begin. Inherent in these transcriptional cycles are (i) functional redundancy, where multiple protein complexes act sequentially to promote each stage in the cycle (Metivier, 2003) and (ii) limitation to the action of estrogen through a restriction in the duration that either ER α or polymerase act on an individual promoter (Reid, 2002; Reid, 2003; Metivier, 2003). This latter restriction ensures that estrogen responsive promoters continuously respond to fluctuations in the level of E2.

At least two different and perhaps complementary mechanisms contribute to the limitation of estrogen signaling. One mechanism involves targeting of ER α for proteasomal degradation concomitant with transcription (Metivier *et al.*, 2003;Reid *et al.*, 2003;Metivier *et al.*, 2004). This involves the cyclical recruitment of potential E3 ligases to the promoter and ubiquitination of ER α (Reid, 2003). Also, a molecular chaperone complex consisting of heat shock proteins (HSPs) may contribute to clearance of the steroid receptor and associated proteins from the target promoter (Freeman and Yamamoto, 2002). Interestingly, components of the proteasome and of the HSP complex are recruited to the promoter at the end of each cycle (Reid *et al.*, 2003).

ER α can access a target promoter in two ways: either through direct interaction with DNA or indirectly by docking onto proteins already bound to DNA, for example with

the AP-1 complex bound to responsive promoters (figure 4). Although the mechanism of transcriptional regulation through indirect promoter binding has not been studied in detail, it is believed to take involve the recruitment of coregulators and eventually the transcriptional machinery, as with ER α associating to a cognate DNA element (Jakacka et al., 2001;Shang and Brown, 2002).

3.2. Regulation of ER α action

The transcriptional output of ER α can be regulated at multiple levels as summarized in figure 4. This section far from being exhaustive describes a few examples of regulation of ER α.

ER α protein level

Regulation of ER α activity can be achieved by regulation of its expression. The levels of ER α are, to certain limits, directly related to its activity (Webb et al., 1992;Lopez et al., 1999). The response to a given amount of ER α also depends on the profile of interacting proteins within cell types and is, as a result, cell type dependent (Webb *et al.*, 1992). Regulation of ER α expression will be detailed later in the introduction.

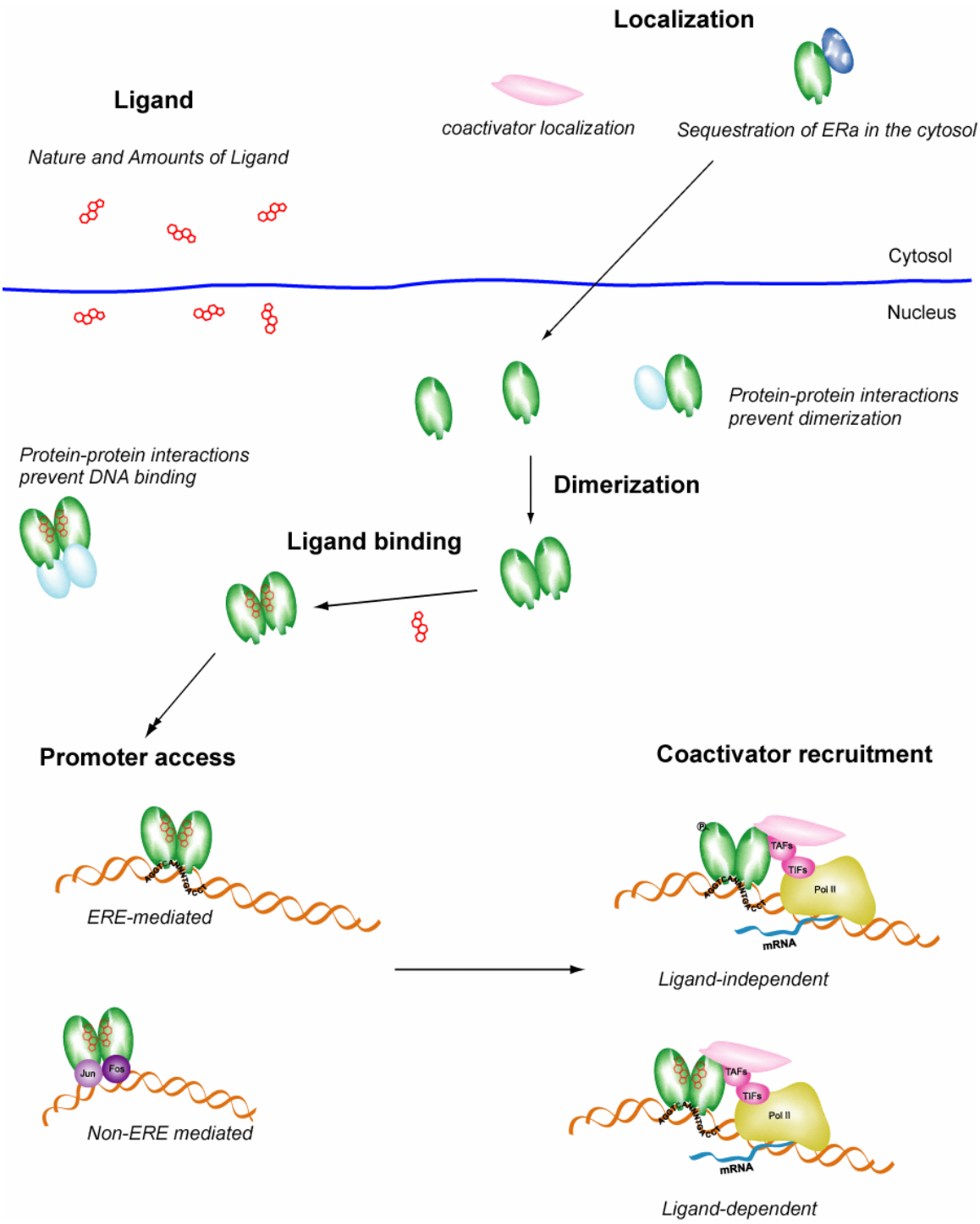


Figure 4. Regulation of ER α action

Examples of steps where ER α action can be modulated starting with localization of the receptor to dimerization ligand binding and promoter binding. All these steps have been shown to be affected under specific cellular condicitons and affect the transcriptional output of ER α.

Localization

To achieve a direct effect on transcription ER α needs to locate to the nucleus. Following the model for glucocorticoid receptor action, it was and still is generally believed that nuclear receptors, including ER α, are located in the cytoplasm prior to ligand activation and that they translocate to the nucleus thereupon. It has repeatedly been shown that ER is located mostly if not exclusively in the nucleus whether or not complexed to its ligand.

The localization of ER α however, is open to regulation. For instance a variant of the metastatic tumour antigen 1 (MTA1) lacking a nuclear localization signal, was found to sequester ER α in the cytoplasm of MCF7 cells (Kumar et al., 2002). This MTA1 variant interacts directly with the AF2 of ER α through a LxxLL NR box like motif (Kumar *et al.*, 2002). Posttranslational modifications of ER α have also been reported to affect localization. Phosphorylation of threonine 311, located in the NLS of ER α, by p38 MAPK, promotes nuclear localization (Lee and Bai, 2002). This data indicate that ER α is normally found in the cell nucleus, although, in certain cellular contexts, it can be present in the cytoplasm. Interestingly, localization of coactivator and corepressors is also the target of regulation and affects ER α transcriptional activity (Baek and Rosenfeld, 2004).

Dimerization

Although the ER α DBD can bind to DNA as a monomer with low affinity *in vitro* (Kumar *et al.*, 1988), ER α exclusively binds DNA as a dimer *in vivo* (Lees et al., 1990). Ligand binding was shown not to be strictly required for dimerization, at least in mammalian cells (Zhuang et al., 1995). However dimer formation of ER α LBD *in vitro* is stabilized by ligands (Tamrazi et al., 2002).

Protein-protein interactions impact on dimerization; it was, for instance, reported that the orphan nuclear receptors TR2 and 4 (testicular orphan receptor 2, and 4) dimerize with ER α. Because TR2 and 4 recognize different DNA sequences they prevent binding of the heterodimer to an ERE (Shyr et al., 2002;Hu et al., 2002). Posttranslational modification of ER α can also affect dimerization. Phosphorylation of serine 239 was shown for instance to regulate dimerization (Chen *et al.*, 1999).

DNA binding

Perfect EREs are rarely found in estrogen responsive promoters. Several sequences give rise to estrogen responsivity to a promoter, ranging from perfectly palindromic EREs, an imperfect palindrome or even a half ERE. Most estrogen responsive genes contain EREs that are mostly imperfect (O'Lone et al., 2004). Although there is no linear correlation, the binding affinity of ER and its transcriptional output are linked in a given cellular environment (Klinge, 2001;Klinge et al., 2001). DNA binding is not strictly dependent on ligand but interaction with DNA is stabilized by ligand binding (Zhuang *et al.*, 1995). Although fairly stable *in vitro*, association of nuclear receptor with DNA appears to be transient *in vivo* as monitored by ChIP and microscopy (McNally *et al.*, 2000;Reid *et al.*, 2003).

High mobility group (HMG) proteins directly contact steroid receptors, and by doing so facilitate and stabilize the interaction between the receptor and its response element, allowing binding to imperfect palindromes or half binding sites (Melvin et al., 2004). Moreover, ER α synergizes with SP1 proteins to bind to an ERE half site when an SP1 site is located nearby (Porter et al., 1997;Safe and Kim, 2004). Often more than one ERE-like sequence is present in an estrogen responsive promoter. The

binding of ER α is cooperative since several EREs synergize to allow ER binding (Klinge, 2001).

Ligand Binding

Ligand binding is a prerequisite in most cases for transcriptional activation, as ligand induced conformation stabilizes a conformation that interacts with coactivators. Ligand binding is also regulated. Association of ER α with an ERE stabilizes the ligand-ER interaction (Klinge, 1999). Protein-protein interactions also affect ligand binding. Coactivators stabilize the interaction of ER α with its ligand (Gee et al., 1999;Watkins et al., 2003). The heat shock protein HSP90 and the HSP90 associated protein p23 also sensitize ER α to estradiol binding (Knoblauch and Garabedian, 1999;Fliss et al., 2000). Finally, Tyrsoine 537 is involved in ligand binding, although it is not sure if this particular phosphorylation is indeed implicated in stabilization of ligand association (Arnold et al., 1997).

Coactivator recruitment

Coactivator recruitment is necessary for ER α action as coactivators mediate chromatin remodeling and the eventual recruitment of the transcription machinery. The main determinant of coactivator binding is the generation of surfaces on ER α that coactivators can interact with, which is promoted by binding of ligand. However many other criteria influence the recruitment of coactivators to a given promoter. The DBD signals allosterically to the LBD and, as a result, the sequence of the ERE bound influences the strength and nature of the interaction of coactivators with nuclear receptors (Wood et al., 2001;Hall et al., 2002).

Posttranslational modifications of ER α also modify coactivator interactions. Indeed, phosphorylation of serine 118 potentiates the interaction of ER α with the AF-1 specific coactivators p68 and p72 (Endoh *et al.*, 1999;Watanabe *et al.*, 2001). Moreover, coactivators can be themselves subject to regulation. For instance SRC3/AIB1, a member of the p160 coactivator family, is phosphorylated in response to extracellular signals and this phosphorylation is necessary for its coactivator function with ER α (Wu et al., 2004). Phosphorylation of SRC3/AIB1 was also shown to relocate the coactivator to the nucleus, were it efficiently interacts with nuclear receptors (Wu et al., 2002).

Other protein interactions can interfere with the binding to coactivators. A decoy coactivator, REA (repressor of estrogen receptor activity), can bind to liganded ER α and compete with SRC-1 preventing its recruitment (Montano et al., 1999). Likewise, coactivators can also compete with LxxLL-motif-containing orphan nuclear receptors SHP and DAX1, which bind the liganded ER LBD but do not have histone modifying activities and thus inhibit ER transactivation (Zhang et al., 2000;Johansson et al., 2000).

Differential recruitment of coactivators by AF-1 and AF-2

As mentioned previously, two distinct activation functions are responsible for the transcriptional activation achieved by ER α. The two AFs are very different in nature but rely on the recruitment of chromatin remodeling coactivators to function. The activity of the two AFs however are cell context and promoter dependent (Tora *et al.*, 1989b;Berry *et al.*, 1990;Metzger *et al.*, 1995;Merot et al., 2004). A given context can be AF-1 permissive, AF-2 permissive or allow both functions to transactivate.

To assess the AF permissiveness of a given cell and promoter context, truncated ERs lacking either AF function are used. Such studies determined a good correlation between an AF-1 permissive context and a context in which tamoxifen works as an agonist (Berry *et al.*, 1990). This situation arises as tamoxifen, while able to inactivate AF-2 through the recruitment of corepressors, activates AF-1 by preventing the repressive interaction between the A and E domains (Metivier *et al.*, 2002b). Consequently, cellular or promoter contexts where tamoxifen functions as an agonist attests to an AF-1 context.

One ER dimer can accommodate one molecule of coactivator or one corepressor, with association mutually exclusive for each dimer (Margeat et al., 2001;Germain et al., 2002). As AF-2 complexed with tamoxifen can bind corepressors at AF-2 or coactivators at AF-1, competition occurs between coactivators and corepressors in binding to tamoxifen-liganded ER α. When excess coactivator over corepressor is present, tamoxifen-liganded ER α dimers preferentially associate with coactivator, whereas in a context where corepressors are in excess, an ER α dimer will more likely bind corepressor. This model has been confirmed experimentally, where it was shown that the over-expression of SRC-1 in cells with an AF-2 context transformed tamoxifen from an antagonist into an agonist (Smith *et al.*, 1997;Shang *et al.*, 2002;Fujita et al., 2002). Likewise, increasing or decreasing the levels or binding of the corepressors N-CoR or SMRT results in a respective decrease or increase of the agonistic activity of tamoxifen (Lavinsky et al., 1998;Fujita et al., 2003).

The agonist activity of tamoxifen can also be modulated through phosphorylation of ER α. Phosphorylation of serine 305 by PKA is absolutely required to allow tamoxifen to induce agonistic activity, even in the presence of elevated levels of coactivators (Michalides *et al.*, 2004).

Although certain coactivators associate with both AF-1 and AF-2, some coactivators, for example p68, are AF-1 specific. Although these coactivators are ubiquitously present in cells their recruitment is enhanced when ER α is phosphorylated on serine 118 (Endoh *et al.*, 1999;Watanabe *et al.*, 2001). However, no strict correlation between the phosphorylation status of ER α and the AF permissiveness has been established. In conclusion, the activity of the two AFs in ER α are regulated through coregulator expression or availability and by post-translational modifications.

Ligand independent recruitment of coactivators: ligand-independent activity

Treatment of cells grown in the absence of ligand with dopamine, IGF-1and EGF results in activation of ER α (Smith et al., 1993;Bunone et al., 1996;Ignar-Trowbridge et al., 1996). An intact AF-1 is required for ligand independent activation of ER α (Bunone *et al.*, 1996;Ignar-Trowbridge *et al.*, 1996), which results from phosphorylation of the receptor. In response to extracellular signals, ER α becomes phosphorylated on several residues (Ali et al., 1993). Phosphorylation of serine 118, by MAPK, activates the receptor in a ligand independent manner, through recruitment of coactivators to AF-1 (Endoh *et al.*, 1999;Metivier et al., 2002a;Deblois and Giguere, 2003;Dutertre and Smith, 2003c). Furthermore, the cyclin A/CDK2 complex phosphorylates serine 104 and serine 106, resulting in an enhancement of the activity of ER α in the presence and absence of ligand (Rogatsky et al., 1999).

Other transcription factors, exemplified by cyclin D1 and the activated dioxin receptor, bind to unliganded receptor and recruit coactivators, thereby conferring ligand independency on ER α (Zwijsen et al., 1998;Ohtake et al., 2003). Finally,

overexpression of coactivators, such as SRC-1, also induces ligand-independent activation of ER α (Kalkhoven et al., 1998).

As previously discussed, deletion of the A domain, which contains an intramolecular corepressor, allows recruitment of coactivator complexes to ER in AF-1 permissive cell contexts (Metivier et al., 2002d). It is therefore also possible that regulation of the interaction between the A and E domains results in ligand-independent activity of ER α.

This notwithstanding, it is important to note that in the majority of studies conducted it is not possible to rule out the possibility that increases observed in transcriptional activity results from an increase in sensitivity to residual estrogens present in the growth medium, rather than a genuine ligand-independent activity. ER α is sensitive to low concentrations of estrogens and for that matter, it is known that estrogenic compounds can contaminate growth media for both yeast and mammalian cells (Mattick et al., 1997;Liu and Picard, 1998).

4. Genomic organization and expression of ER α

4.1. Genomic organization of the ER α gene.

Our appreciation of the complexity of the ER α gene unit continues to increase as time passes. Since the cloning of the first human ER α cDNA in 1986 (Green *et al.*, 1986;Greene *et al.*, 1986), numerous cDNA variants, divergent in their 5' untranslated region (UTR), have been identified. This reflects alternative promoter usage and extends the number of exons associated with the generation of ER α mRNA. The availability of the human genome sequence has revealed that the promoter regions

associated with the ER α gene now spans ~ 300 kb, instead of the 140 kb initially thought (Kos et al., 2001). Most exons and their associated promoters, including upstream non-coding exons, are conserved between man and mouse and extend over a similar genomic stretch (figure 5B; Kos *et al.*, 2001;Swope et al., 2002).

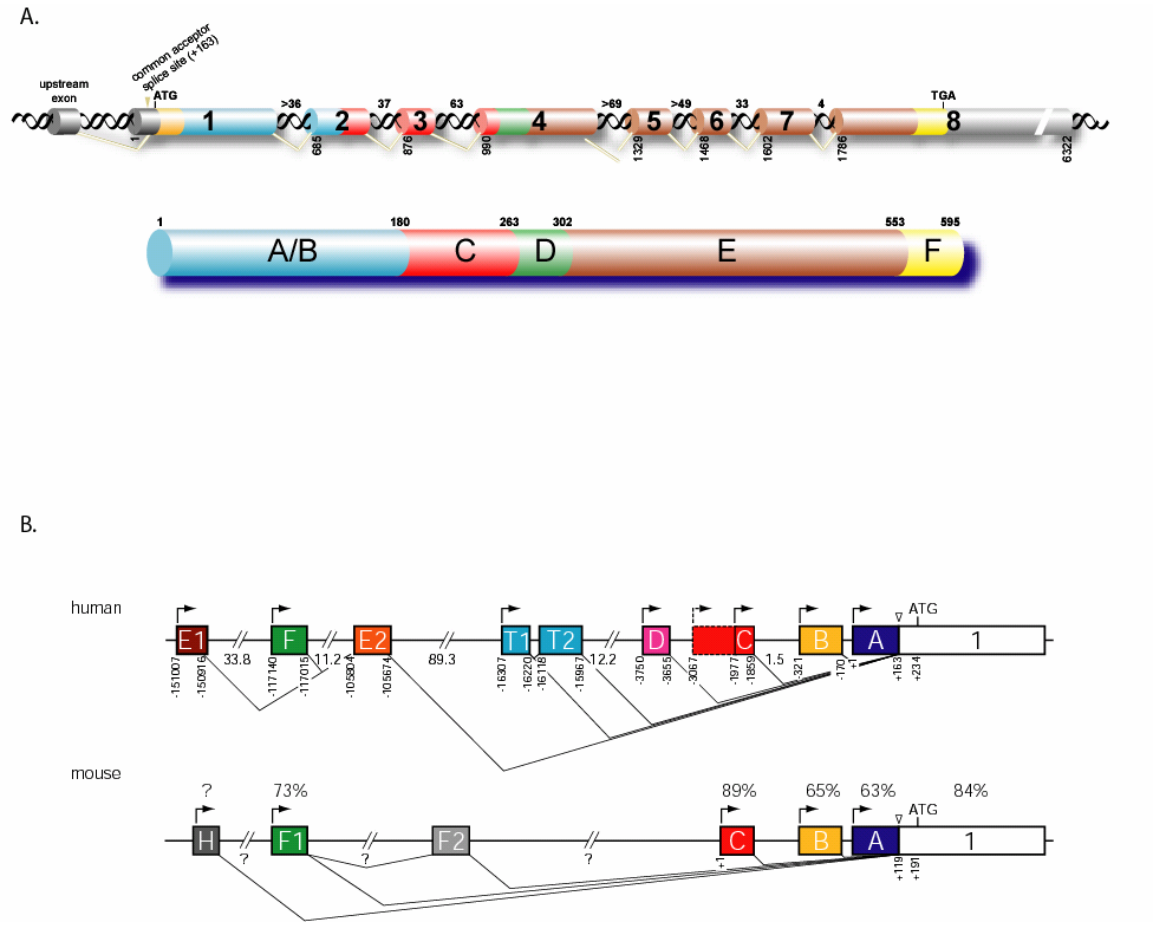


Figure 5 Genomic organization of the ER α gene

A. The coding region of ER α spans 8 exons. B. Both mouse and human ER α genes comprise multiple 5' untranslated exons. All these exons splice to a common acceptor splice site in exon one either directly or indirectly. Most of them are conserved between the two species.

The coding sequence of ER α comprises 8 exons, numbered 1 to 8 (figure 5A). Upstream exons are designated with a letter (figure 5B). These 5' non-coding exons generally splice to a common acceptor splice site located in exon 1. The most

proximal exon, exon A, is a component of exon 1. Further complexity exists with exons E1 and F, as they initially splice to exon E2 prior to splicing to the common splice site. Consequently, the resulting 5' non-coding sequence is a hybrid between two or three non coding exons; the very 5' sequence arising from promoter activity, a middle sequence derived from exon E2 and the 3' sequence downstream from the common splice acceptor site of exon1. A similar situation occurs in mouse where exon F1 the ortholog of exon F splices to exon F2 before it slices to the common splice site (Kos *et al.*, 2001).

4.2. Multiple promoters and the transcriptional regulation of ER α expression

Very little is known about the promoters both in terms of organization and regulation. Most ER α promoters do not display any obvious TATA box, CCAAT box, or GC box, although the testis specific promoter T1, contains all of these features (Brand et al., 2002). As a result, the start of transcription for each variant is relatively loose and ER α promoters tend to show a very weak activity in transcription assays (Kos *et al.*, 2001 ; our own unpublished data).

Two studies have compared the expression of different variants in different tissues. Although a direct quantitative methodology of promoter usage was not employed in these studies, it was found that most promoters were used in most tissues tested (Flouriot et al., 1998;Kos et al., 2000). Apart from the E promoter in human or the H promoter in mouse which seem to be specifically employed in liver, the other variants show very little specificity (Flouriot *et al.*, 1998;Kos *et al.*, 2000). Interestingly, it was also found that variants detected in the same tissues were different between mouse and human. The A and B variants, which are the most abundant variants expressed in

human tissues, are absent from mouse tissues, with the most abundant in mouse being the C variant.

In human A, B, C and F mRNA variants are regulated by E2, either in a positive or negative way, depending on cell type (Donaghue et al., 1999;Denger et al., 2001). In mammary epithelial cells, it was shown that the transcription factor AP2 gamma plays an essential role in ER α expression (Schuur et al., 2001). In MCF-7 cells, Akt kinase signals to the ER α B promoter, through negative regulation of the transcription factor FOXO3a to downregulate ER α expression (Guo and Sonenshein, 2004). However, despite these tantalizing reports, little is known of the extracellular signals, the promoters or transcription factors that direct cell-type specific expression of ER α. Moreover, in all studies on ER α promoter activity, weak intrinsic expression compromises detailed analysis of effects.

4.3. Functional implications of alternative splicing and alternative promoter usage

Almost every possibility of alternative splicing of ER α has been detected at the level of mRNA(Lu et al., 1999;Hirata et al., 2003). Alternative splicing and exon skipping of non-coding upstream exons and coding exons are known to occur. Relatively few of the resulting transcripts can be translated into a functional protein. Accordingly, despite the plethora of mRNA transcripts, only few proteins counterparts are known to exist.

In human however it was found that upstream exons E and F can not only splice to the common acceptor splice site of exon 1 but also to the acceptor splice site of exon 2 (Flouriot et al., 2000). The variant generated can be translated into a 46 KDa protein using two AUGs present in exon 2. These AUGs are present in a good Kozak

sequence for the promotion of ribosome assembly. The corresponding protein has the C-terminal sequence of the full-length ER α but lacks the first 173 amino acids, including AF-1. As a result this ER α isoform exhibits dominant negative activities in cells where the AF-1 of ER α is used (Flouriot *et al.*, 2000).

4.4. ER α protein turnover

Every protein in the cell is subject to renewal and constant degradation. Degradation of short-lived molecules like transcription factors is often effected by the ubiquitin-proteasome pathway (Ciechanover et al., 1984;Pickart, 2004). ER α is no exception to the rule and is also ubiquitinated and degraded by the proteasome (Alarid et al., 1999;El Khissiin and Leclercq, 1999;Nawaz et al., 1999). Ligand binding can incredibly shorten the half-life of ER α and accordingly increases receptor ubiquitination (Nawaz *et al.*, 1999;Reid *et al.*, 2003). Ligand-induced proteasome degradation is linked to transcription as transcription is required for ligand to target the receptor for proteasomal degradation (Lonard et al., 2000;Reid *et al.*, 2003).

Every nuclear receptor analyzed so far is downregulated in a protesome-dependent manner after ligand activation (Zhu et al., 1999;Hauser et al., 2000;Lin et al., 2002;Blanquart et al., 2004). Likewise transcription factors are also targeted for proteasomal degradation upon activation by phosphorylation (Fuchs et al., 2000;Bauer et al., 2002). This would point out towards a general mechanism coupling transcription and degradation.

A common machinery is involved in the addition of a ubiquitin chain to a protein target. Only the E3 ligase is believed to be specific to the substrate and recognizes the target to be ubiquitinated (Pickart, 2004). No E3 ligase of ER α has been identified to date. However MDM2 was shown to catalyse the ubiquitination of the related

androgen receptor (Lin *et al.*, 2002). In any case it seems unlikely that only one E3 ligase regulates ubiquitination of ER α. The analysis of the turnover of p53 reveals that many E3 ligases can regulate the turnover of a given transcription factor (Dornan et al., 2004).

The ER α antagonist ICI 182,780 can not only compete with estradiol for binding to the LBD, thereby preventing DNA binding and transcriptional activation but was also found to potently decrease the half life of ER α by targeting the receptor to proteasomal degradation (Dauvois et al., 1992;Wijayaratne and McDonnell, 2001). The fact that ICI 182,780 does not allow ER α to transactivate but still targets the receptor for proteasomal degradation which would suggest that at least two distinct mechanisms of turnover exist (Wijayaratne *et al.*, 2001).

Extracellular signals and signal transduction pathways were reported to affect proteasomal degradation of ER α. TGF β was shown to decrease ER α protein stability in a proteasome dependent manner in breast cancer cell lines (Petrel and Brueggemeier, 2003). Also, the kinase ERK 7 stimulates proteasomal degradation of ER α (Henrich et al., 2003). Conversely, activation of PKA by the Akt/Pi3K pathway protects the receptor from ligand-induced degradation (Marsaud et al., 2003;Tsai et al., 2004).

5. Physiology of estrogens

5.1. Ligand nature and availability

Recruitment of coactivators occurs when ER α is complexed to one of its agonistic ligands. However, the levels of estrogens are not constant in an organism and vary

greatly throughout time. As a result ER α is not activated to the same extent sensing the variations in plasma E2 levels.

The primary source of E2 in females is the ovary. Synthesis of E2 occurs from the aromatisation of androgens by the enzyme P450 Aromatase. Aromatase activity has also been detected in extragonadal tissues and most notably in the adipose tissue. Extragonadal sites would be the most important source of E2 in males and in females after the menopause when ovaries stop producing E2 (Simpson et al., 2000). Although there are several different estrogens produced by the body, including estrone and estriol as well as E2, they all act as agonists although they bind ER α with different affinities (Kuiper et al., 1997).

The plasma levels of estrogens increase at puberty and vary thereupon throughout the menstrual cycle, estrogen levels being highest prior to ovulation (Gruber et al., 2002). At the menopause, depletion of the ovarian follicles leads to a steady decline in ovarian E2 production. In the male, although estrogens also play a physiological role nothing is known of the regulation of E2 production by extragonadal tissues.

5.2. Estrogen function

Deletion of both ERs or of aromatase that synthesizes estrogens from androgens has allowed a detailed characterization of estrogen function. The sterility phenotype of ER and aromatase knockouts confirmed the importance of estrogens in the reproductive function (Dupont *et al.*, 2000). Estrogens however are not involved in morphogenesis or sex determination. Both male and female knockout mice develop normally up to puberty (Dupont *et al.*, 2000). Differentiation of the reproductive tract is impaired in mice deficient for either ER but no other obvious phenotype could then be characterized.

At the menopause estrogen levels drop dramatically and is concomitant with several dysfunctions in non-reproductive tissues. Also, the existence of gender specific diseases not related to the reproductive function pointed out that estrogens could have a broad effect on non-reproductive function. Non-reproductive tissues affected by estrogens include most notably the brain, the vascular system and bone (Manolagas and Kousteni, 2001).

Using mouse ER deficient mice, it was determined that most responses to estrogens are mediated by ER α. ER β plays a compensatory role in the ovary and in the osteoportective action of estrogens in females, and only shows an idiosyncratic function in the response to anxiety in female mice (Krezel et al., 2001).

5.3. Tissue distribution of estrogen receptors

The use of high activity radiolabeled estradiol allowed the identification of the main estrogen binding tissues. These corresponded to the main responsive tissues namely the uterus, the vagina and the anterior pituitary (Jensen *et al.*, 1962;Jensen and DeSombre, 1972). Since then, a multitude of approaches have been used to detect ERs in tissues (Warner et al., 2003). The discovery of ER β prompted the comparison of the tissue distribution of the two ERs (Kuiper *et al.*, 1996;Kuiper et al., 1997). This was done with sensitive techniques compared to the use of radiolabeled ligands 10-20 years earlier and led to the identifcation of the ER protein or its mRNA in a plethora of tissues (Kuiper *et al.*, 1997;Couse et al., 1997;Lemmen et al., 1999). In fact there might not be a single tissue or cell type that has not been shown to express either or both ERs.

In addition, several transgenic mice strains containing a reporter gene under the transcriptional control of an ERE were developed (Nagel et al., 2001;Ciana et al.,

2001;Toda et al., 2004;Lemmen et al., 2004). This approach allowed the identification of tissues in which ER α or ER β can be activated by its ligand and affect transcription. This method confirmed that many tissues are able to respond transcriptionally to estrogens consistent with the expression pattern of both ERs. These studies were however very divergent regarding the nature of the estrogen responsive tissues, questioning the relevance of these results.

5.4. Non-reproductive sites of action: the example of bone

Bone Physiology

The skeleton is formed of two different tissues; cartilage and bone. Each tissue has its own specialized cell types: the chondrocyte in cartilage and the osteoblast and osteoclast in bone. Bone is mainly formed of collagen fibers (type I collagen represents 90% of the total protein) that fix hydroxyapatite (calcium phosphate) crystals that compose mineralized bone. At the cellular level, the two cell types present in bone have opposite roles. The osteoblast deposits and mineralizes the bone matrix while osteoclast readsorbs bone. Osteoblasts and osteoclasts originate from different stem cell lineages, with osteoblasts derived from the mesenchymal lineage and osteoclasts belong to the hematopoeitic compartment (Karsenty, 1999). A balance between anabolism and catabolism is required for the maintenance of a healthy bone structure. Osteoblasts and osteoclasts are present in the same environment. Consequently, both cell types communicate to regulate each other's differentiation and function. Bone is a dynamic tissue under constant renewal with new bone being made, opposing the continuous resorption. Local and systemic factors impact on bone

remodeling and affect bone density by regulating the function of either or both osteoblasts and osteoclasts.

Skeletal development starts in the embryo with the formation of mesenchymal condensations that prefigure the future skeletal elements they will later form (Olsen et al., 2000). In the case of intramembranous ossification, cells from the mesenchymal condensations differentiate into osteoblasts that constitute part of the skull and the clavicles. For the other skeletal elements, the mesenchymal cells of the condensations differentiate into chondrocytes that constitute a cartilage anlage of future bones. The anlage becomes vascularized, with chondocytes subsequently replaced by osteoblasts. Bone cells therefore have different developmental origins depending on the skeletal element they construct. Bone cells have however very similar phenotypes independent of which part of the skeleton they come from.

Osteoblast differentiation

Differentiated cells are characterized by a phenotype that mirrors their function. With osteoblasts, the most obvious terminal phenotype is an ability to deposit calcium phosphate crystals. Osteoblast progenitors can be isolated from bone and differentiated *in vitro* in a manner consistent with the *in vivo* process. Perhaps not surprisingly, one of the first extracellular markers present in differentiating osteoblasts is collagen type I, which is laid down as an extra-cellular matrix and constitutes the target structure for subsequent mineralization. Additional markers have been identified; namely alkaline phosphatase (ALP), which appears at the early stages of differentiation, and osteocalcin, which is expressed later, concomitant with mineralization (Owen et al., 1990;Aronow et al., 1990). The function of these two

secreted proteins is not clear but their expression is tightly linked to osteoblastic differentiation and as a result characterizes the osteoblast phenotype.

Differentiation of a progenitor cell requires triggering of specific genetic programme that lead to the expression of proteins required to cell-type specific function. The induction of a genetic programme is controlled by extracellular cues and is transduced by transcription factors. Many transcription factors involved in osteoblast differentiation have been identified although the precise although their functional hierarchy is still poorly understood (Stains and Civitelli, 2003). The transcription factor Cbfa1 is absolutely required for osteoblast differentiation and bone formation. It is also sufficient to induce expression of osteoblast specific genes in ectopic cell types (Ducy et al., 1997). Cbfa1 acts in concert with other osteoblast-specific transcription factors such as osterix to induce osteoblast differentiation (Nakashima et al., 2002).

The expression of CBFa1 is under control of extracellular signals that are affect bone formation Locally produced growth factors play a pivotal function in orchestrating the differentiation of osteoblasts. In particular, BMP's (bone morphogenetic proteins) are the most efficient promoters of osteoblast differentiation known, with BMP-2 stimulating preosteoblast proliferation, extracellular matrix production and eventually mineralization. It does so, at least partly, by stimulating both CBFa1 expression and function (Lee et al., 2000;Lee et al., 2002). Although it exerts complex effects *in vivo*, TGF β potently and consistently inhibits osteoblast differentiation *in vitro* (Centrella et al., 1994;Alliston et al., 2001). Moreover, TGF β promotes the expression of collagen type I by osteoblast precursors but prevents the proceeding of differentiation (Centrella *et al.*, 1994).

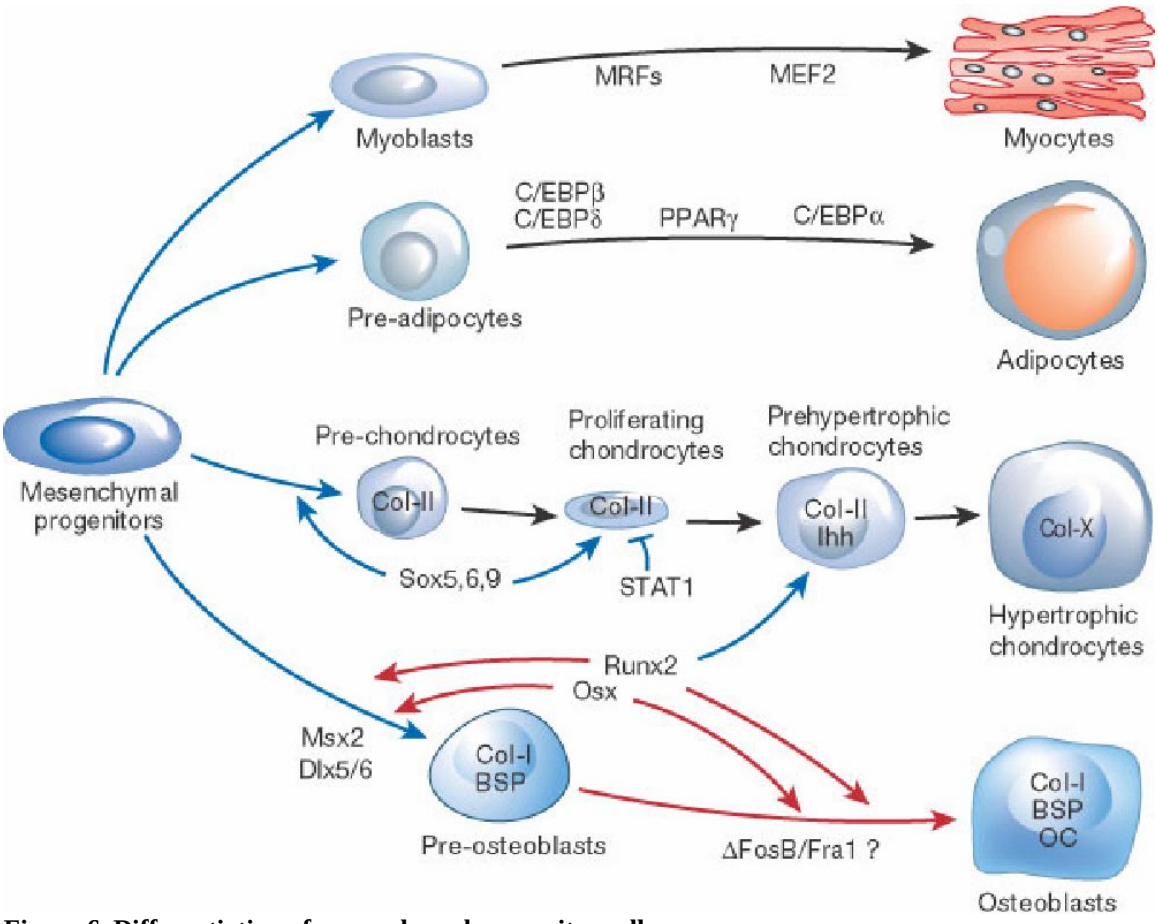


Figure 6. Differentiation of mesenchymal progenitor cells

Mesenchymal cells can differentiate into different cell types. Differentiation triggers the expression of a genetic programme characterized by the expression of lineage specific transcription factors leading to the expression of the differentiated phenotype. Myoblasts fuse to form multinucleated myocytes, adipocytes store lipid drolplets, chondorcytes deposit a collagen type X extracellular matrix while osteoblasts deposit a collagen type I matrix (adapted from Stains *et al.*, 2003)

Osteoblasts arise from mesenchymal stem cells. These cells, when cultured *ex vivo* have the potential to differentiate into numerous cell types (figure 6). With different extracellular cues, mesenchymal stem cells can differentiate into myocytes, adipocytes, chondrocytes and osteoblasts. Differentiation is however exclusive and a cells do not usually expresses markers of two different cell types. Undifferentiated osteoblast progenitors have low levels of transcription factors involved in the

differentiation of myocytes, adipocytes, and osteoblasts (Garcia et al., 2002). With differentiation, osteoblasts suppress expression of markers of other cell types (Garcia *et al.*, 2002). Osteoblast and adipocyte differentiation is exclusive with suppression of adipocyte differentiation resulting in an increase in osteoblast differentiation (Akune et al., 2004).

Members of the TGF β family of cytokines are important regulators of mesenchyaml cell differentiation. Among them, the BMPs (bone morphogenetic proteins) are potent promoters of the osteoblast phenotype. BMP2 not only increases the differentiation of osteoblast but also directs multipotential mesenchymal cells towards the osteoblast lineage (Katagiri et al., 1994;Katagiri et al., 1997). The action of BMP2 on osteoblasts is mediated by Cbfa1 (Lee et al., 2003). On the other hand TGF β prevents the expression of osteoblast specific genes like ALP and osteocalcin although it promotes expression of collagen type I (Centrella *et al.*, 1994;Alliston *et al.*, 2001). It is also a general inhibitor of mesenchymal cells, preventing differentiation of adipocytes and myoblasts (Choy et al., 2000;Liu et al., 2001).

Estrogens and bone

After menopause, the levels of serum sex steroids, including estrogens, become reduced. This reduction in estrogen levels is associated with a time dependent decrease in bone density, that can eventually result in osteoporosis (Riggs et al., 2002). Postmenopausal bone loss can often be improved by estrogen treatment (Lindsay et al., 1976;Riggs *et al.*, 2002). This effect made it clear that estrogens are involved in bone homeostasis. Additionally, gonadectomy in rodents is able to reproduce bone loss observed in postmenopausal osteoporosis, and is also prevented by estrogen replacement. Gonadectomy was thereafter used as a model for

osteoporosis. Both menopause and gonadectomy result in an increase in osteoclast and osteoblast activity with an imbalance towards resorption, progressively resulting in bone loss. This increase in bone turnover is ameliorated by estrogen treatment, which suppresses the activity of both osteoclasts and osteoblasts (Riggs *et al.*, 2002).

The function of estrogens and their receptors in bone homeostasis has been further investigated using knock-out mouse models deficient either in the estrogen synthesizing enzyme aromatase or in ER α or β (Oz et al., 2000;Sims et al., 2002;Sims et al., 2003). The loss of both receptors or of aromatase in female mice mimicked the effects of ovariectomy (Oz *et al.*, 2000;Sims *et al.*, 2002;Sims *et al.*, 2003). In female, single ER knockout mice loose bone after ovariectomy, whereas the double knockout shows a reduced bone density that is not further decreased by ovariectomy. This suggests that the maintenance of bone density is mediated by the two ERs in female (Sims *et al.*, 2003). In male orchidectomy also induces bone loss but the phenotypes are complicated by the involvement of androgens and the androgen receptor as both androgens and estrogens are important for bone maintenance (Sims *et al.*, 2003). Upon overiectomy or orchidectomy, both osteoblasts and osteoclasts are activated increasing bone trunover. Estrogen treatment of overiectomized or orchidectomized mice results in a decrease in activity of both osteoblasts and osteoclasts. This osteoprotective effect is mediated essentially by ER α in female mice, ER β playing a small redundant role (Sims *et al.*, 2003). In males, only ER α mediates the osteoprotective effect of estrogens (Sims *et al.*, 2003). However it is not possible from these studies to know whether the osteoprotective action of estrogens affects bone directly or if intermediary systems are involved. In this regard, targeted, cell type specific knockouts will be very informative to determine the cell types mediating this effect.

At high doses, estrogens not only prevent gonadectomy-induced bone loss but also act to increase bone density, even in intact animals. This anabolic response to estrogens solely relies on ER α in both males and in females (McDougall et al., 2002;McDougall et al., 2003). This effect, as opposed to osteoprotection, is mostly mediated by an increase in osteoblast function (Samuels et al., 1999). Although cell-type specific knockouts would confirm whether this effect is direct or indirect, an earlier study indicates that the anabolic effect of high estrogen doses is direct, as it affects bone density locally (Takano-Yamamoto and Rodan, 1990). However it might not be possible to extrapolate from this result to mice as the osteogenic response in rats is achieved with lower concentrations of estrogens as opposed to the supraphysiological concentrations needed in mice to elicit this response (Turner, 1999). As a result the osteogenic response in mouse and rat might occur through distinct processes.

Estrogens and osteoblasts

There is considerable debate about the expression of functional estrogen receptors in osteoblasts. Although estrogen binding sites have been reported (Komm et al., 1988;Eriksen et al., 1988), the number of binding sites in osteoblasts are however very low ranging from a few hundred to a thousand sites per cell, as compared to known estrogen target tissues like uterus which comprise 15,000 sites/cell (Eriksen *et al.*, 1988;Davis et al., 1994). Still, the number of binding sites in osteoblasts is significantly higher than in fibroblasts (Eriksen *et al.*, 1988). In addition to the full length receptor, the N-terminally truncated 46 KDa isoform of ER α was detected in primary human osteoblasts at similar levels as full length receptor (Denger *et al.*, 2001). The receptors present in osteoblast are anticipated to be functional and

correspondingly could activate an exogenous estrogen responsive reporter gene (Ernst et al., 1991). It is not clear however whether the expression level of ER α in osteoblasts would be sufficient to allow the mediation of estrogen effects *in vivo* (Karsenty, 1999).

ER α mRNA was found to be upregulated on development of the osteoblast phenotype (Bodine et al., 1998;Wiren et al., 2002;Bonnelye and Aubin, 2002). This observation indicated that ER α is expressed specifically in osteoblasts and that its expression is part of the osteoblast differentiation programme. The involvement of ER α in osteoblast function was further pointed out by the fact that it can interact with the osteoblast transcription factor Cbfa1, thereby enhancing its transcription activation (McCarthy et al., 2003).

In terms of response estrogens conflicting reports exist. Estrogens could slightly enhance differentiation of osteoblasts cultured *ex vivo* while others report that estrogens had no effects on osteoblast differentiation (Keeting et al., 1991;Scheven et al., 1992;Qu et al., 1998). Estrogens have also been reported to promote osteoblast differentiation of mesenchymal stem cells as opposed to adipocytic differentiation (Dang et al., 2002;Okazaki et al., 2002).

Although ER α is present in osteoblasts and estrogens can have a mild effect on osteoblast differentiation *ex vivo* it is not sure to what extent these results are physiologically relevant. It is also not sure whether ER α expression is specific to osteoblast considering the broad expression pattern that has been described.

Objectives

Since their involvement in post-menopausal osteoporosis has been identified, estrogens have been shown to be crucial for bone homeostasis. Estrogens can affect both osteoblast and osteoclast function in the bone environment. ER α is present in osteoblasts and it was suggested to affect directly osteoblast growth and function.

ER α expression is also upreglated during osteoblast differentiation and it can interact with osteoblast specific transcription factors. This made it clear, that ER α was as a transcription factor involved in osteoblast function.

Nothing was known about the specificity and regulation of the 7 ER α promoters. osteoblasts presented the unique advantage of keeping their phenotype *in vitro* and of growing as a very homogeneous cell population. As a result, osteoblasts would have been ideal to examine the specificity of ER α promoter(s) usage. Confident that ER α expression was under the control of osteoblast specific factors, I was therefore planning to identify the promoter(s) responsible for osteoblast specific expression to then further study their regulation.

I planned to develop an *in vitro* assay for osteoblast differentiation using cell lines for molecular studies and confirm the results obtained with mouse primary osteoblasts. I then wanted to develop assays that would allow detection of ER α mRNA, protein and activity in osteoblasts. To control for the specificity of ER α promoters in osteoblasts, I also wanted to use the ability of mesenchymal stem cells to differentiate into other cell types.

As desribed below, as I developed the experimental system, many surprises came along that challenged many of the beliefs I had when I started this project.

Results

1. *In vitro* differentiation of an osteoblastic cell line and of primary osteoblasts

Several tissue culture systems have been developed to study osteoblast differentiation *in vitro*. Several osteogenic cell lines are available and it is also possible to work with primary cells, which can be obtained from newborn mouse calvaria by serial enzymatic digestions or flushed from the bone marrow of new born and adult mice.

Committed osteogenic cells can develop the osteoblast phenotype *in vitro* with increasing cell contacts (Owen et al., 1990). A few days after confluency has been reached, osteogenic cells increase production of collagen type I, which constitutes the basic extracellular component of bone matrix. The deposition of a collagen matrix is followed by the deposition of non-collagenous proteins like alkaline phospahtase (ALP) and osteocalcin (Owen *et al.*, 1990). The addition of ascorbic acid and of a phosphate source such as β-glycerophosphate allows osteoblasts to mineraliz the depositied collagen matrix (Owen *et al.*, 1990;Aronow et al., 1990).

Three histochemical markers, followed over time, were used to determine the purity of cultured osteoblasts and their ability to differentiate. ALP, an early differentiation marker, was monitored using a colorimetric assay. Additionally, the Van Giesson and Von Kossa assays were used to stain the extracellular collagen matrix and mineralization respectively.

The three cellular systems mentioned earlier, available to study osteoblast differentiation *in vitro* were evaluated for their ability to grow and differentiate in vitro. The isolation of mouse bone marrow mesenchymal stem cells from adult mice proved difficult. Cellular survival was low as was the purity of cultures as determined

by alkaline phosphatase expression (data not shown). Consequently, this procedure was not used.

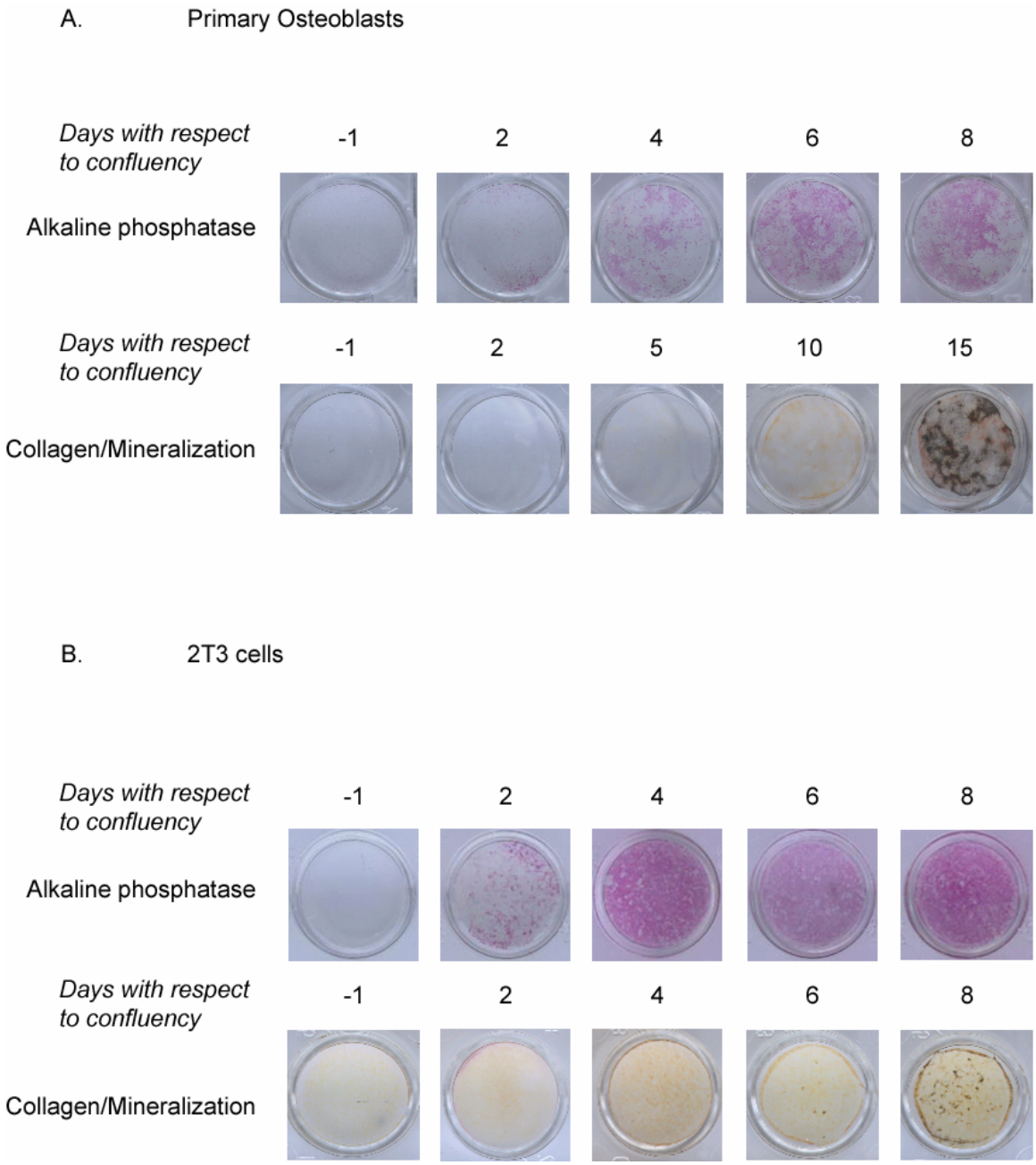


Figure 7. *In vitro* differentiation of mouse primary osteoblasts and of 2T3 cells

A. Primary mouse osteoblasts and B. 2T3 cells were monitored for differentiation using three different stains. The ALP is stained in purple. The orange Van Giesson stain shows the extracellular collagen matrix while the silver nitrate Von Kossa assay stains in black mineralized bone nodules. Differentiation occurs with growing cells contacts and is first characterized by the apparition of ALP positive cells. The deposition of the collagen matrix is followed by its mineralization.

Mouse calvarial osteoblasts have been extensively used *ex vivo* to study the genetics of osteoblasts as they recapitulate the *in vivo* differentiation process. Indeed, a bone phenotype resulting from a cell-autonomous osteoblast defect, is usually mirrored by deficient *in vitro* differentiation of calvarial osteoblasts (Li et al., 2000;Kim et al., 2003;Kenner et al., 2004). The development of the osteoblastic phenotype in mouse calvarial osteoblasts is shown in figure 7A. Alkaline phosphatase expression was first detected 2 days after confluency was achieved. Most cells became alkaline phosphatase positive 8 days after confluency. Addition of β-glycerophosphate and ascorbic acid allowed the cells to mineralize the collagen matrix. The first collagen nodules (orange stain) were readily detectable 4 days post confluency. The matrix became more visible by day 10 and became mineralized 15 days after confluency, as detected by the dark silver staining.

Osteogenic cell lines are more amenable than primary osteoblasts to generate the number of cells required for molecular studies; moreover, they can easily be transfected. The mouse 2T3 cell line has been shown to function like primary calvarial osteoblasts, and differentiates into osteoblasts after confluency is reached (Ghosh-Choudhury et al., 1996). The kinetics of differentiation and mineralization of the 2T3 line were assessed, as previously described for primary osteoblasts (figure 7B). ALP positive cells were first detected 2 days after confluency. At day 4, most cells were ALP positive and remained positive throughout the assay period. Collagen nodules were first detectable at day 2 and became mineralized from day 8 with mineralization increasing to day 10.

These results indicate that both 2T3 cells and primary calvarial cells differentiate into mineralizing osteoblasts *in vitro*. 2T3 cells, however, are entirely homogeneous, synchronized and differentiate to osteoblasts more quickly. In light of these

observations, we therefore chose to utilize the 2T3 cell line preferentially in the following experiments but to verify key results, when possible, with primary osteoblasts, to exclude artifacts arising from use of an immortalized cell line.

2. ER alpha expression in osteoblasts

2.1. Characterisation of ER α protein isoforms in osteoblasts

The mouse ER α gene generates multiple mRNAs and protein products through alternative promoter usage and alternative splicing (Kos *et al.*, 2000). In addition to the full-length ER α of 66 KDa, shorter isoforms have been characterized both at the protein and mRNA levels. Notably, two in frame ATGs located in exon 2, generate a 46 KDa protein. In man, this isoform is produced through splicing of exon F to exon 2 and also by internal ribosome entry (Barraille et al., 1999;Flouriot *et al.*, 2000). Interestingly, expression of ER α 46 in human primary osteoblasts occurs at the same level as full-length ER α (Denger *et al.*, 2001). Although the 46 KDa protein isoform of ER α has never been detected in mouse, its corresponding transcript has been identified and the two ATGs are conserved between human and mouse exon 2 (Denger *et al.*, 2001).

To evaluate whether ER α or any of its shorter isoforms were expressed in osteoblasts, western blot analysis was performed using a mouse specific ER α C-terminal antibody (MC-20) of cell extracts prepared from differentiated ALP positive (6 days post-confluency) 2T3 cells. Mouse uterus cell extracts that contain high levels of ER α and *in vitro* translated mouse ER α (mER α) 66 kDa and 46 KDa were used as controls. In order to identify non-specific immunoreactive products, cell extracts from the murine

fibroblastic NIH 3T3 cell line were also analysed. NIH 3T3 cells are ER α negative and do not support expression of estrogen responsive reporter constructs (Castoria et al., 1999).

Three major immunoreactive species were detected by MC-20 in 2T3 cell extracts (figure 8A and B). A doublet running approximately at the same size as full-length ER α as compared to *in vitro* translated full length mER α or from uterus extracts, with another band migrating above 49 KDa. A very faint product migrated at the same size as the in vitro translated 46 KDa isoform of mER α (figure 8A). The product migrating above 49 KDa is unlikely to be derived from mER α as it is present in the ER α negative NIH 3T3 cells.

ER α specifically associates with agonists (e.g. β-2-estradiol (E2)) and antagonists (e.g. ICI 187,780) in the ligand binding domain, located in the C-terminal half of the protein. Binding of either E2 or ICI 187,780 specifically targets the receptor for proteasomal degradation and decreases receptor half-life from 4-5 h in the absence of ligand to ~3 h on binding of E2 and to 30 min in the presence of ICI 187,780 (Dauvois *et al.*, 1992;Wijayaratne *et al.*, 2001). This property was used to ascertain that the bands observed corresponded to ER α. Figure 8B shows that in 2T3 cells, treatment with E2 or with ICI 182,780 for 24 h induced a reduction in the doublet at 66 KDa. The unspecific lower product was however not affected by any of the treatments showing that this effect is specific. As previously reported (Alarid *et al.*, 1999;El Khissiin *et al.*, 1999;Nawaz *et al.*, 1999), co-treatment with the proteasome inhibitor MG 132 blocked downregulation of ER α by E2 and ICI 182,780 (figure 8B). This indicates that ligand-induced downregulation of the 66 KDa doublet is indeed dependent on proteasome activity.

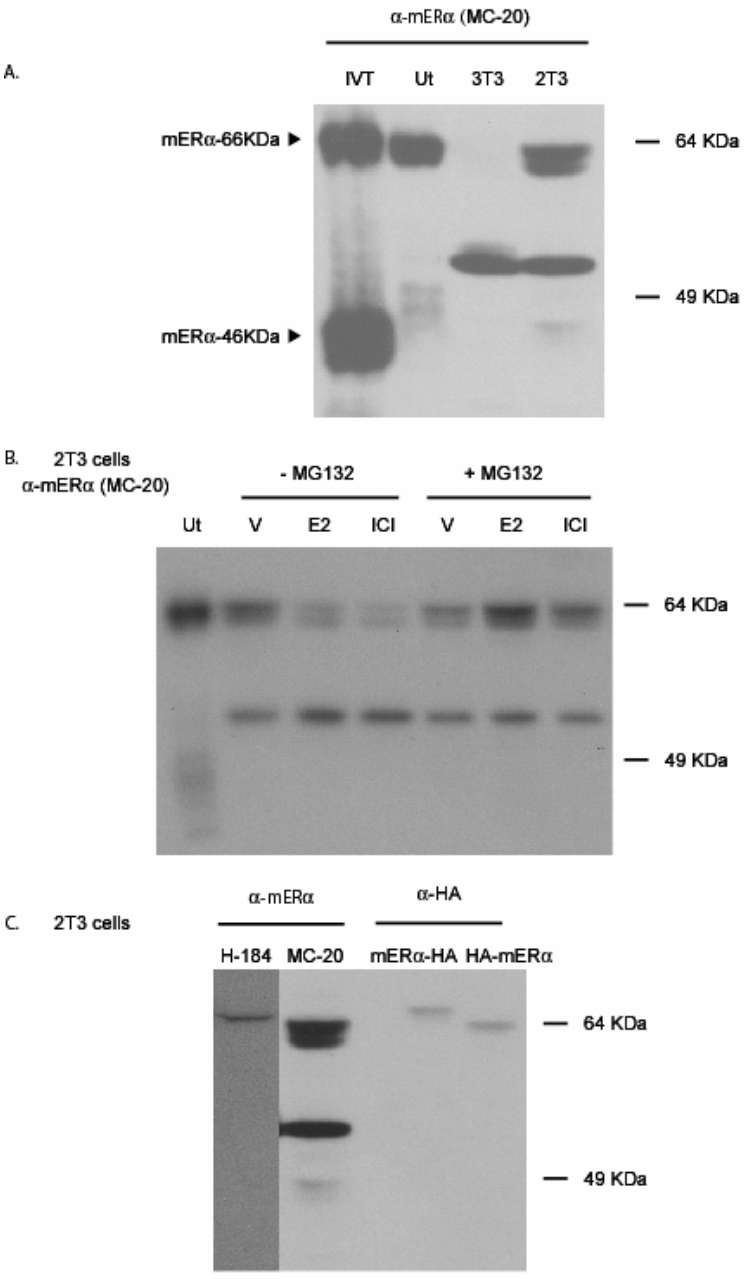


Figure 8. Western blot analysis of mER α in osteoblasts

A. western blot analysis of in vitro translated (IVT) mER α 46 and 66 KDa isforoms, mouse uterus protein extracts (Ut), mER α negative NIH 3T3 cells and differentiated day 6 2T3 cells. **B.** Western blot analysis of mER α in 2T3 cells treated with E2 and the antagonist ICI 182,780 (ICI) run alongside uterus extracts (Ut). Ligand-induced degradation was blocked using the proteasome inhibitor MG 132. Equal cell number (600,000) was loaded in each lane for 2T3 cell extracts **C.** Western blot analysis of endogenous mER α in day 6 2T3 cells with the N-terminal H-184 antibody and the C-terminal MC-20 antibody. Transfected HA-tagged mER α was detected using an HA antibody (H-11). The 64 and 49 KDa marks correspond to marker sizes.

The two bands migrating at 66 KDa may arise either from different transcripts or by post-translational modification. This was addressed by expressing epitope tagged transgenes of ER α in 2T3 cells. If post-translational modification gives rise to the different migratory forms of ER α, the tagged version should also appear as two forms. Conversely, the occurrence of a single migratory epitope tagged species would indicate that alternative transcripts or alternative translational events give rise to two species of slightly different molecular weight. Constructs expressing ER α, tagged N or C-terminally with a single HA epitope, were transfected into 2T3 cells, which were then analyzed 6 days after confluency occured. The two constructs had the same transactivation ability as wild type (WT) mER α showing that the tags did not affect receptor function (data not shown). In contrast to endogenous ER α, expression of the tagged ER α in day 6 2T3 cells did not exhibit dimorphism (figure 8C). In addition, we used an antibody (H-184) raised against the first 184 amino acids of ER α. This antibody detected only one immunoreactive band by western blot with differentiated 2T3 samples (figure 8C).

Together, these results indicate that ER α is expressed in mouse osteoblasts in two forms whose size is around 66 KDa. Both species have biochemical characteristics of ER α as they are specifically degraded by the proteasome after treatment with ER specific ligands. As only one of the two species reacts with an N-terminal antibody the unreactive species would differ with its N-terminus from full length ER α. The difference in migration is unlikely to be due to post-translational modification of the full-length receptor, as transfected HA-tagged ER α appeared as a single migratory form. These results suggest that osteoblasts express two isoforms of ER α that are of a similar size as known full-length ER α but would differ in their N-termini. The 46 KDa isoform of ER α that was previously detected in human osteoblasts was not

readily detectable in mouse osteoblasts and is unlikely to play a significant role as compared to the other forms of mER α.

2.2. The expression of ER α in osteoblasts is not sex specific

Estrogens and their receptors are generally regarded as female specific. However, in non-reproductive tissues, such as bone, the effects of estrogens do not depend on the sex of the animal (McDougall *et al.*, 2002;McDougall *et al.*, 2003;Sims *et al.*, 2003). We confirmed this observation through comparing the expression of ER α in differentiated male and female primary osteoblasts. Male osteoblasts did not differ phenotypically from female osteoblasts and had similar differentiation kinetics *in vitro* (data not shown). As shown in figure 9, no difference exists in ER α expression between male and female osteoblasts, indicating that the expression of ER α in osteoblasts is not sex specific.

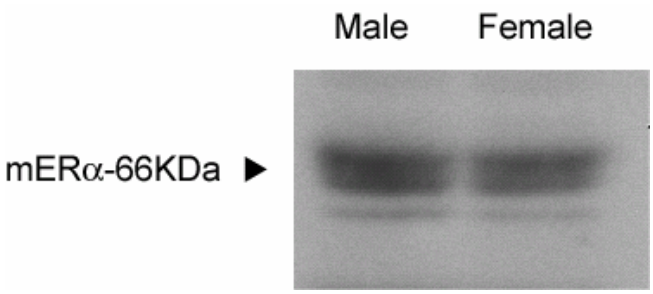


Figure 9. Expression of mER α in male and female mouse primary osteoblasts.
Western blot analysis of mER α using the C-terminal antibody MC-20. Protein extracts were obtained from *in vitro* differentiated male and female primary osteoblasts 10 days after confluency. 600,000 cells were loaded in each well.

2.3. ER α expression in osteoblasts is low compared to uterus

One function of estrogens is to induce endometrial proliferation prior to implantation of fertilized eggs. Uterus is a direct target tissue of estrogens and ER α is absolutely required for this process (Dupont *et al.*, 2000). Consequently, large amounts of ER α are present in the different cell types of the uterus (Jensen *et al.*, 1972). The comparative level of ER α expression in extracts prepared from female mouse osteoblasts and from mouse uterus was estimated by western blot analysis. Loading was normalized to DNA content rather than protein content, thereby ensuring that identical cell numbers were analysed in this comparison. As shown in figure 10, ER α expression is, although detectable, negligible in osteoblasts as compared to the large quantity detected in uterus.

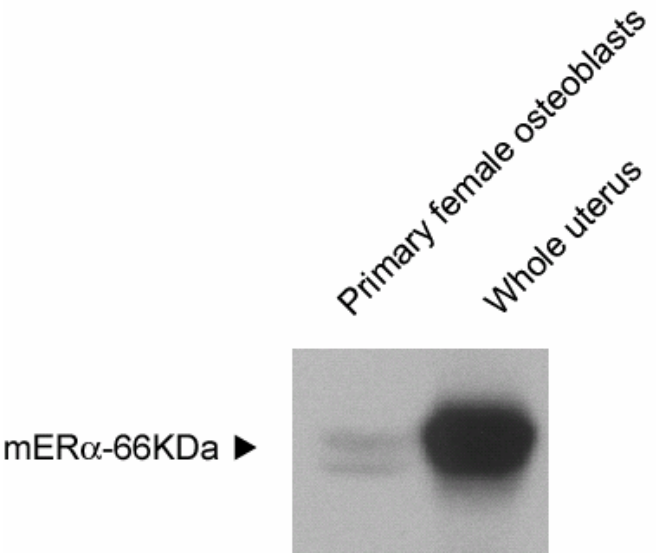


Figure 10. Comparative expression of mER α in osteoblasts and in uterus

Osteoblast protein extracts were from differentiated female primary osteoblasts 10 days postconfluency. Whole Uterus were dissected from 6 day-old female mice and protein extract prepared from them. 100 µg of osteoblast protein were loaded as reference. The amounts of samples loaded were normalized to DNA content to insure that each well contained the same number of cells.

3. ER alpha expression during osteoblast differentiation

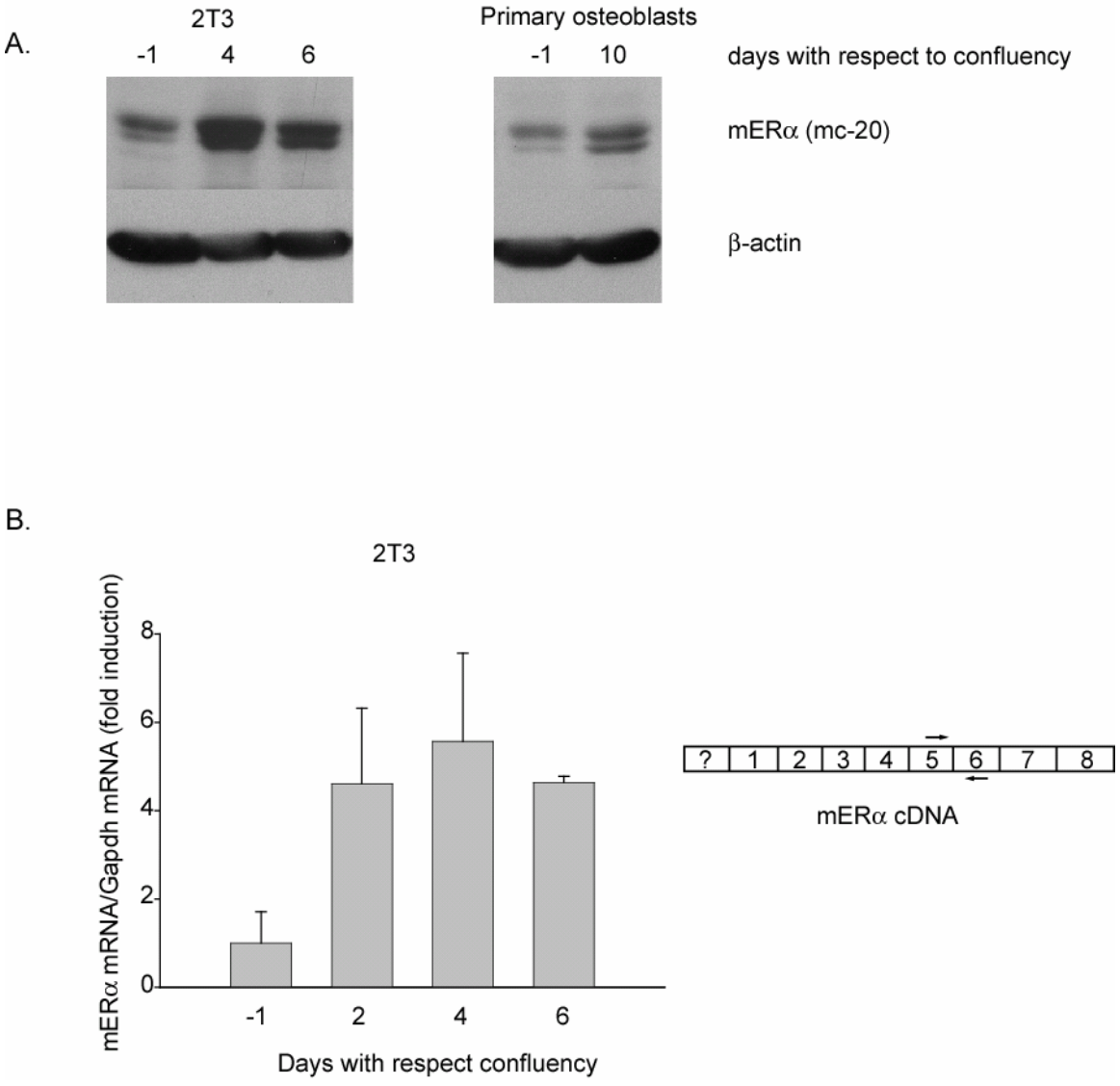
3.1. ER α expression increases with osteoblast differentiation

The onset of osteoblast differentiation reflects the activation of a genetic programme in which transcription factors and structural proteins are sequentially induced. One of the earliest events, is the apparition of the transcription factor Cbfa1 which triggers the ordered expression of the osteoblast phenotype together with other transcription factors (Stains *et al.*, 2003). Interestingly, in rat and man, ER α protein levels were also reported to increase with osteoblast differentiation, raising the possibility that ER α expression is integrated in an osteoblast specific differentiation programme (Arts et al., 1997;Bodine *et al.*, 1998;Wiren *et al.*, 2002).

We therefore wanted to evaluate the kinetics of ER α expression throughout the differentiation process and functional activity of osteoblast derived from animals or from 2T3 cell line. Total protein was isolated from 2T3 cells one day before confluency and 4 and 6 days post confluency and one day before confluency and 10 days postconfluency for primary osteoblasts. Protein prepared from an equal number of cells was loaded with β-actin used as a loading control. 2T3 and primary osteoblast protein extracts contain the same immunoreactive doublet at 66 KDa (figure 11A). The ER α doublet was visible in undifferentiated subconfluent primary osteblasts and 2T3 cells. In 2T3 cells, expression of the mER α doublet increased from day -1 to day 4 slightly decreasing at day 6. Increase in mER α expression also occurred between day -1 and day 10.

To determine if increased ER α protein correlates with increased mRNA expression, total RNA from undifferentiated and differentiated 2T3 cells, one day prior to

confluency and 2,4 and 6 days after confluency, was isolated and quantitative RT-PCR performed using primers encompassing exons 5 and 6 (figure 11B). ER α mRNA does indeed increase with differentiation, with a 5-fold increase in ER α mRNA occurring, paralleling increased protein levels.



Figrue 11. mER α expression during osteoblast differentiation

A. Western blot analysis of mER α during osteoblas differentiation. Equal number of cells (600,000) was loaded per well and even loading was controlled with an anti-β-actin antibody. mER α protein expression was assessed with MC-20 in undifferentiated and differentiated 2T3 cells. Day –1 2T3 cells and primary osteoblast are ALP negative while day 4 and 6 2T3 cells and day 10 are mostly ALP positive. B. RT-PCR analysis of mER α mRNA expression throughout osteoblast differentiation of 2T3 cells. Primers were located in exon 5 and 6. Expression of mER α transcript increased during differentiation as opposed to expression of GAPDH transcript.

In conclusion, ER α expression increases with osteoblastic differentiation at the mRNA and protein levels. Expression of both protein isoforms at 66 KDa is upregulated with differentiation. It is however, not possible to say if upregulation is a direct consequence of differentiation or if the observed increase arises from non-specific effects, such as the increased cell contact that occurs between cells as confluency is achieved.

3.2. Inhibiting osteoblast differentiation with TGF β does not impair ER α expression

To distinguish between the possibilities that ER α is specifically up-regulated during osteoblast differentiation and that it is regulated by other unspecific signals, we made use of the impact TGF β has on osteoblast differentiation. TGF β plays indeed a central role in the regulation of osteoblast differentiation. Although it increases type I collagen production in prevents terminal differentiation and expression of ALP and osteocalcin (Centrella *et al.*, 1994;Alliston *et al.*, 2001;Sowa et al., 2002). TGF β however does not promote the differentiation of mesenchymal cells into other cell types, but acts as a general inhibitor of mesenchymal cell differentiation (Choy et al., 2000). Although the action of TGF β on proliferation varies, depending on the origin of the osteogenic cells, there appears to be no direct connection between the effects of TGF β on differentiation and proliferation (Centrella *et al.*, 1994). As TGF β is able to specifically target differentiation, it can be used to dissociate the development of the osteoblast phenotype from the increase in cell contacts and thereby determine if ER α expression is linked to differentiation.

As seen on figure 12A, ALP expression and mineralization of 2T3 cells are both inhibited after continuous treatment with TGF β. In contrast, a single 24 hours of TGF

β treatment did not affect ALP expression in day 6 osteoblasts (figure 12A). To determine the effect of TGF β on 2T3 cell proliferation, cell number was assessed by quantifying DNA amounts. Although continuous TGF β treatment slightly increased cell numbers in the early stages of culture, it did not have any significant effect on cell number in postconfluent cells (figure 12B).

The effect of TGF β treatment on the expression of ER α was then determined. To differentiate between possible direct effects of TGF β on ER α expression and the effects linked to TGF β induced changes in the differentiation status of the cells, protein were isolated both from 24h treated cells and from continuously treated cells. As seen earlier, ER α expression increased between day –2 and day 6 with respect to confluency (figure 12C). TGF β had indeed an effect on mER α protein expression that was unrelated to osteoblast differentiation. A 24h treatment of subconfluent 2T3 cells or 6 days postconfluency induced a downregualtion of ER α (figure 12C). Continuous TGF β treatment for 6 days however did not affect ER α expression (figure 12C). Although differentiation is blocked with continuous (6 days) TGF β treatment there is no variation in ER α expression.

The analysis of these results is complicated by the fact that ER α expression is the target of TGF β independently of osteoblast differentiation. Still, expression of mER α increases from day -1 to day 10 whether or not the cells are continuously treated with TGF β, that is whether the cells are ALP positive or not. As a result we can conclude that ER α expression does not correlate with the expression ALP, raising the possibility that ER α expression is not linked to osteoblast differentiation.

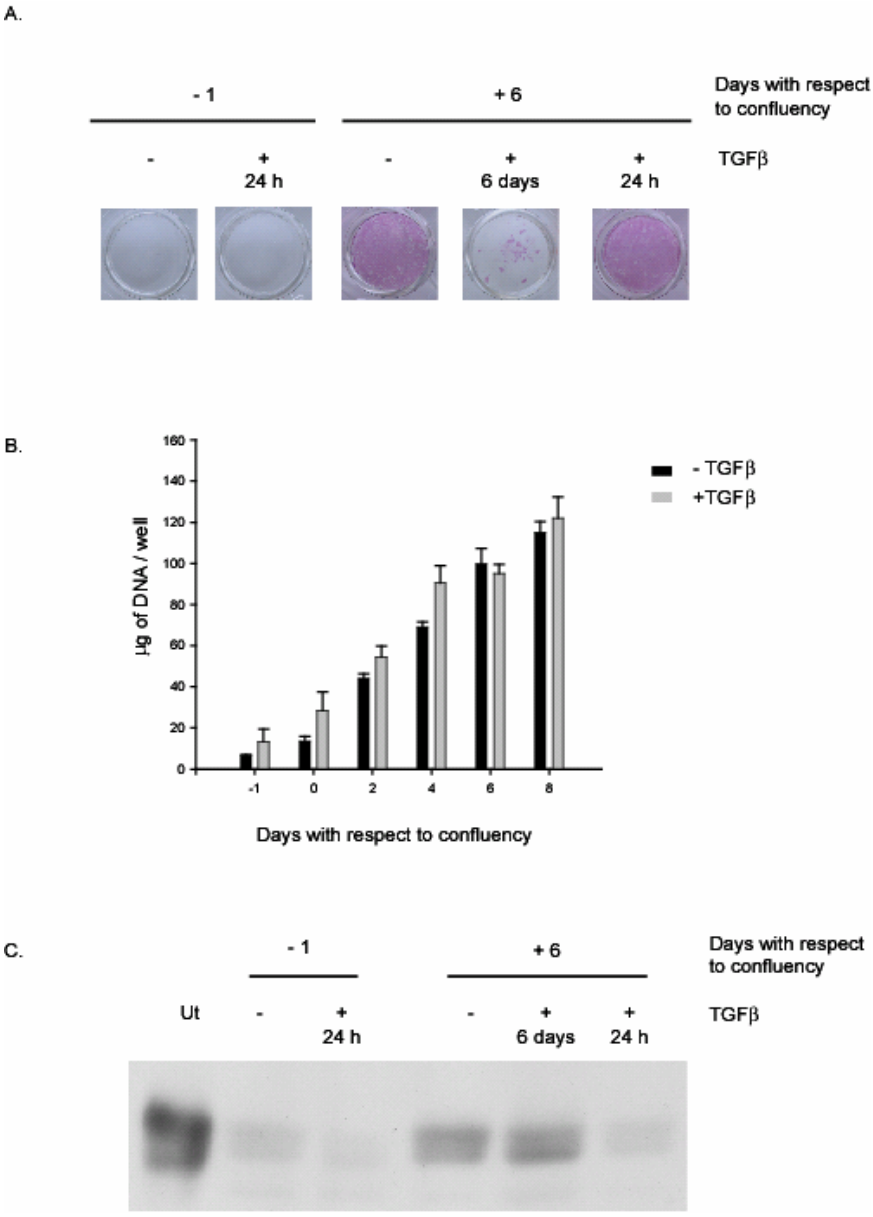


Figure 12. Inhibition of osteoblast differentiation with TGF β does not affect ER α expression

A. Differentiating 2T3 cells in the presence or absence of TGF β were submitted to a colorimetric ALP assay. Subconfluent undifferentiated 2T3 cells (-1) do not express ALP. At day 6 2T3 cells are ALP positive (+6), continuous TGF β (5 ng/ml) treament (+ 6 days) inhibits ALP expression. A transient 24 hr TGF β treatment (+ 24h) does not affect ALP expression. **B.** Cell number during 2T3 cells osteoblastic differentiation was assessed by quantifying DNA and shows that TGF β does not affect significantly cell number. **C.** Protein were isolated from 2T3 cells one day prior to confluency and 6 days after confluency, run alongside uterus (Ut) extract, and submitted to western blot analysis using MC-20. Equal cell number (600,000) was loaded in each well. Legend is as in A.

TGF β induced down-regulation of endogenous ER α has been previously reported in several breast adenocarcinoma cell lines (Stoica et al., 1997;Petrel *et al.*, 2003). However, this is the first report of TGF β induced down regulation of ER α expression in osteoblasts. This effect will be discussed later in more detail. In contrast, exposure to continuous TGF β treatment that blocks osteoblastic differentiation does not impair ER α expression. The discrepancy between the effect of a single 24h TGF β treatment and that of a continuous treatment can be explained by the fact that TGF β induces inactivation of its receptors and components of its signaling pathways (Zhang and Laiho, 2003;Shi and Massague, 2003). A single TGF β treatment will directly and reversibly downregulate ER α while continuous TGF β prevents osteoblastic differentiation and also inactivates its signalling. As a result, continuous TGF β treatment is ineffective at downregulating ER α.

3.3. ER α expression during osteoblastic and myogenic differentiation of C2C12 cells.

The use of TGF β does not dismiss the possibility that expression of mER α in osteoblasts occurs in the early steps of differentiation prior to ALP expression. To determine whether mER α expression is genuinely osteoblast specific we used the ability of mesenchymal progenitor cells to differentiate into several cell-types. Indeed, pluripotential mesenchymal cells can not only differentiate into osteoblasts but also into adipocytes, myoblasts or chondroblasts (Phinney et al., 1999;Pittenger et al., 1999). C2C12 cells are a well-characterized mesenchymal cell line, which can differentiate into osteoblasts in the presence of BMP2, although they preferentially differentiate into myoblast (Katagiri et al., 1994). High doses of BMP2 are required to

redirect C2C12 cells towards the osteoblastic lineage while they spontaneously fuse into myotubes in low serum medium (Katagiri *et al.*, 1994). The resulting population produces an extracellular collagen matrix and express specific osteoblast differentiation markers, including Cbfa1, ALP and osteocalcin (Katagiri *et al.*, 1994;Lee *et al.*, 2000;Maeda et al., 2004). We therefore used the dual differentiation potential of C2C12 cells to further evaluate the association of ER α expression and osteoblastic differentiation.

C2C12 cells were grown to confluency whereupon the serum content of the growth medium was lowered from 15% to 5%. Under these conditions, C2C12 differentiated spontaneously into multinucleated myotubes. Subconfluent undifferentiated cells were neither multinucleated nor did they express ALP (figure 13). Figure 13A shows that the first multinucleated myotubes appeared 3 days after confluency while most cells had fused into myotubes at day 6. Nonetheless, C2C12 cells induced to differentiate into myoblasts did not express ALP either at day 3 or day 6 (figure 13A). In contrast, the addition of 300 ng/ml BMP2 at confluency induced osteoblastic differentiation. ALP was expressed by most cells at day 3 and day 6 and no multinucleated myoblasts were visible (figure 13A). Transient 24h treatment with BMP 2 did not reverse the formation of myotubes although it induced ALP expression in a few cells (figure 13A).

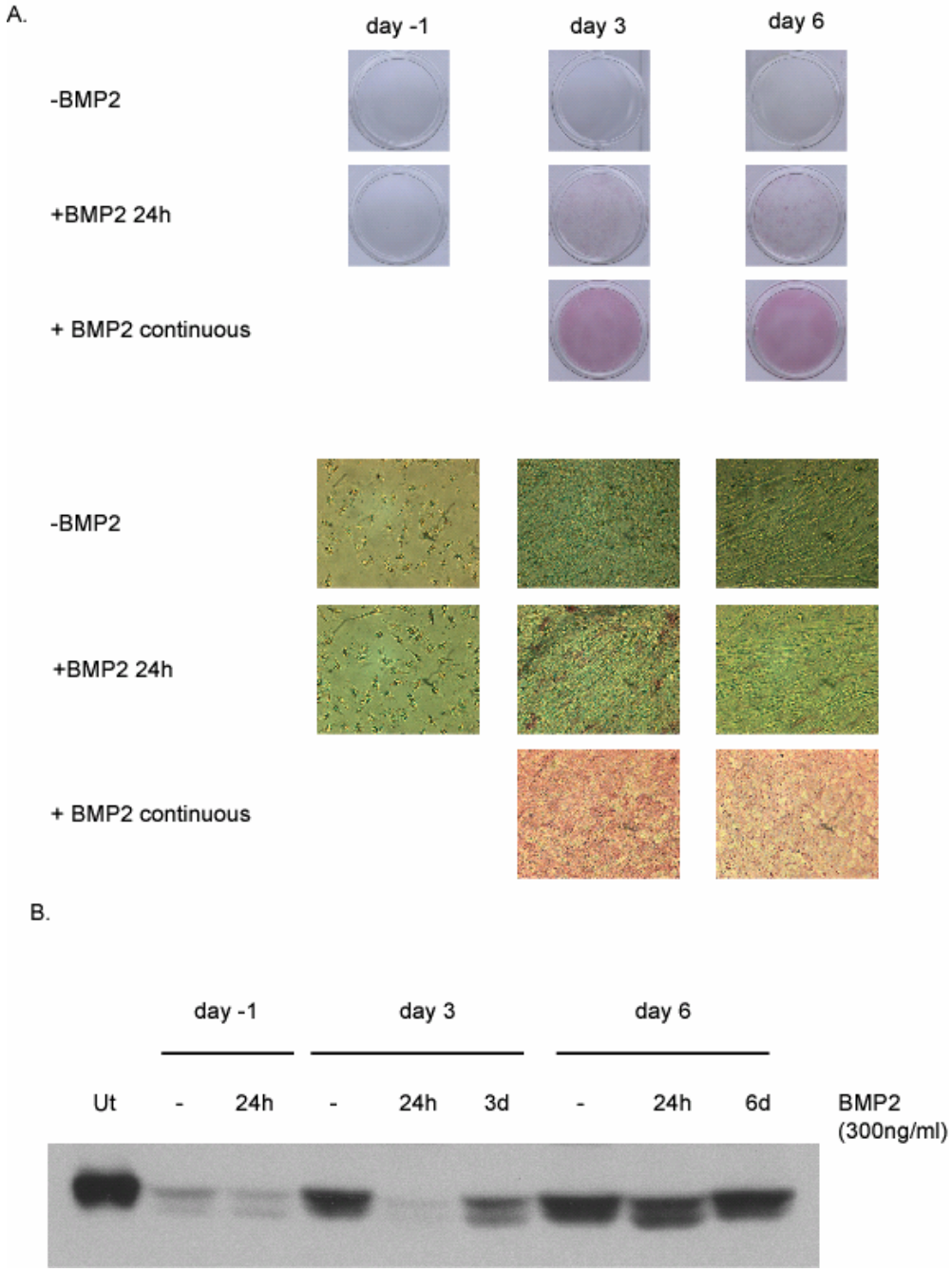


Figure 13. mER α expression during osteoblast and myobast differentiation of C2C12 cells.

A. The upper panel shows ALP assay of differentiating C2C12 cells in the presence or absence of BMP2 (300 ng/ml) up to 6 days after confluency. The lower panel is a close up of the wells presented in the upper panel. In the absence of BMP2, C2C12 cells differentiate into multinucleated myoblasts and do not express ALP. Transient 24h BMP2 treatment at day 3 and day 6 only induces a few cells to express ALP. Continuous BMP2 treatment induces ALP expression and no multinucleated myoblasts are visible. **B.** Western blot analysis of mER α using MC-20. Proteins were isolated at the different stages showed in A. 100 ug of protein loaded in each well for C2C12 cells and run alongside uterus sample (Ut).

ER α was detectable in C2C12 cells. Like for TGF β however, BMP2 treatment induced a decrease in ER α expression that was independent of the differentiation status of C2C12 cells (figure 13B). 24h treatment of C2C12 cells at day -1 and day 3 dramatically decreased the levels of mER α protein, the same occurred at day 6 but to a lesser extent. A three-day continuous treatment with BMP2 was also able to reduce the level of mER α but not as much as a 24h treatment. Most importantly, a 6 day long BMP2 treatment did not affect the levels of mER α as compared to untreated cells. This last result shows that untreated C2C12 cells that have a myoblastic phenotype express as much mER α as ALP positive C2C12 cells. Although the expression of ER α increased between day -1 and day 6, the increase was not a specific consequence of osteoblast differentiation (figure 13B).

As for TGF β, BMP2 is able to downregulate mER α independently of the differentiation phenotype. Similarly, continuous treatment attenuates the BMP2-induced downregulation of ER α as BMP2 is less capable of downregulating mER α after three days of treatment and has no effect after 6 days of continuous treatment. The fact that continuous BMP2 treatment fails to affect mER α expression suggests like for TGF β that BMP2 inactivates its own signalling. BMP2 and TGF β, although they have divergent effects on mesenchymal cell differentiation, belong to the same family of cytokines and have a common mechanism of action and share common effectors (Lee *et al.*, 2002;Lai and Cheng, 2002;Shi *et al.*, 2003).

4. Estrogen receptor transcriptional activity in mouse osteoblasts

4.1. The transcriptional activity of the estrogen receptor increases with differentiation and displays high levels in the absence of ligand

ER α is expressed in osteoblasts, and its expression increases with differentiation (figure 5A). We then wished to define if ER α in osteoblasts is transcriptionally active and if transcriptional activity correlates with expression level.

To monitor mER α transcriptional activity, we first isolated primary mouse osteoblasts from newborn transgenic mice that express β-galactosidase under the control of three tandem EREs (Nagel *et al.*, 2001). The isolated osteoblasts differentiated *in vitro* as monitored by ALP activity, and ER α protein expression increased with differentiation, as observed with wild-type osteoblasts (figure 14A). ß-galactosidase activity was determined in undifferentiated subconfluent and in differentiated osteoblast cell extracts. As ICI 182,780 not only competes with E2 in binding to ER but also targets the receptor for proteasomal degradation, the values obtained were nomalized to that of ICI 182,780 treated cells. Figure 14A shows that in subconfluent cells there is no difference in the induction of β-galactosidase activity in the presence of E2 as compared to ICI 182,780. However, in differentiated primary osteoblasts, a significant increase in activity occurs with both vehicle and E2 treatments, as compared to ICI 182,780 treated cells. Surprisingly, no significant induction of β-galactosidase activity by E2 occurred, as compared to treatment with vehicle alone.

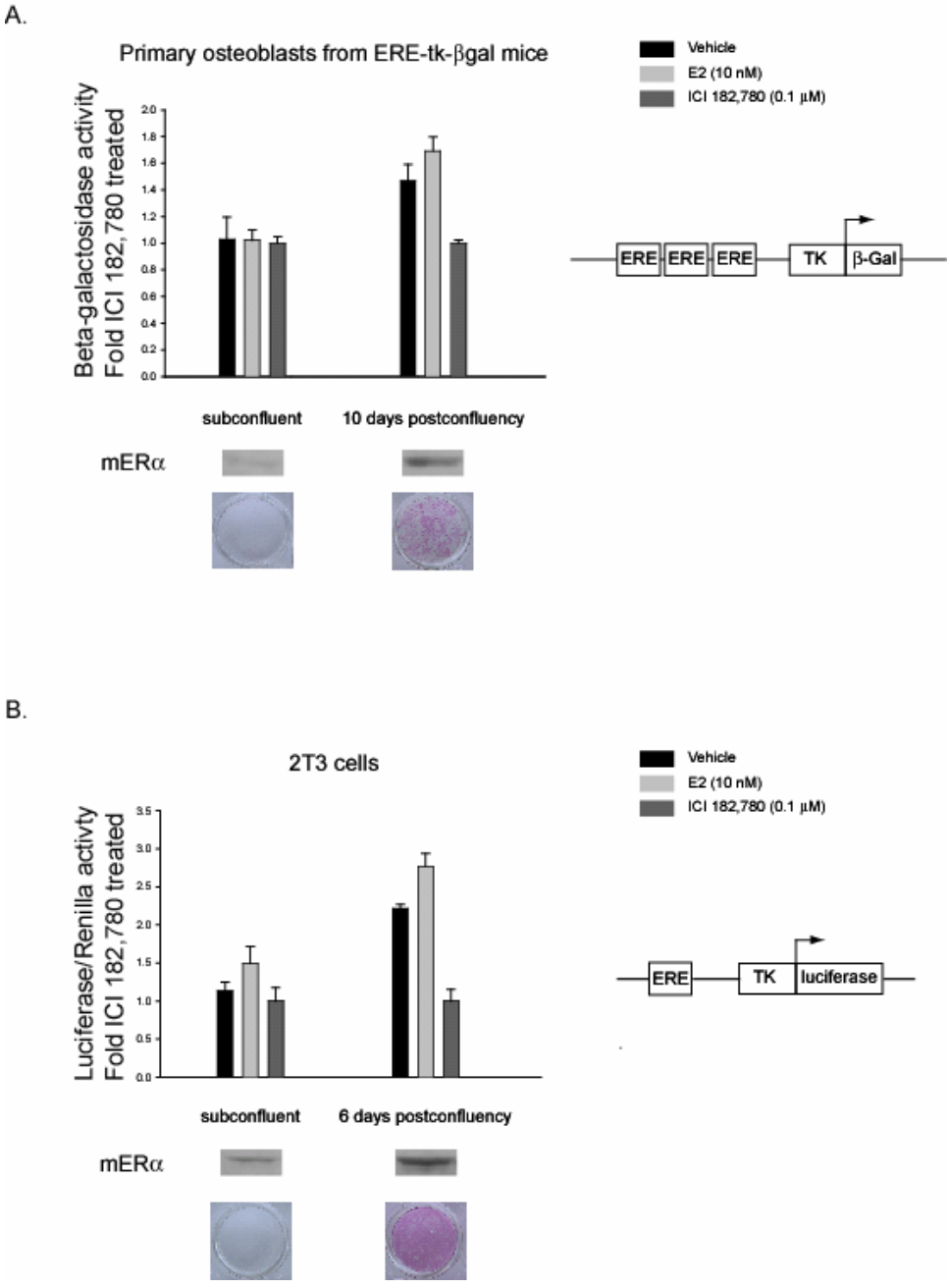


Figure 14. Estrogen receptor transcriptional activity in osteoblasts

Cells were allowed to differentiate in vitro. Protein expression of mER α was assessed by western blotting with the H-184 antibody. The transcriptional output of mER α was assayed for the corresponding reporter after 24h treatment with vehicle, 10 nM E2 or 0.1 μM ICI 182,780. Activity is represented as fold of ICI 182,780 treated cells. **A.** Primary osteoblasts were isolated from transgenic mice containing a β-galactosidase (β-Gal) transgene under the control of 3 EREs and the minimal Tk promoter. Primary cells were analyzed for β-Gal activity at subconfluency or 10 days postconfluency. **B.** 2T3 cells were transfected with the ERE-tk-luciferase reporter 48h before harvest. Cells were analyzed for luciferase activity and activity was normalized to transfection efficiency with renilla activity.

These findings were then confirmed in 2T3 cells, transiently transfected with an estrogen responsive ERE-tk-luciferase construct, either at subconfluency or 5 days post confluency. Luciferase activity was again normalized to the mean values obtained from cells treated with ICI 182,780. As with primary osteoblasts in undifferentiated, subconfluent cells., reporter expression does not increase significantly above that of ICI 182,780 treated cells (figure 14B). In contrast, luciferase activity in differentiated 2T3 cells is significantly higher in both vehicle and E2 conditions than in cells treated with ICI 182,780 (figure 14B).

The increase in transcriptional activity monitored in osteoblasts with respect to differentiation is likely to be consequent on the elevation of mER α levels that occurs. However, ligand dependent steroid receptors are generally not active in the absence of ligand. In contrast, orphan receptors known as estrogen related receptors (ERRs) bind to EREs and activate transcription in the absence of any estrogens. One of them, ERR α has been shown to be active in osteoblasts (Bonnelye et al., 2001). However, because their ligand binding domain cannot accommodate ligand, ERRs do not bind and therefore are not inhibited by ICI 182,780 (Vanacker et al., 1999). The results presented are normalized to activities seen when cells are treated with ICI 182,870, where a significant increase is seen in the absence and presence of E2. ERRs are, as a result, unlikely to be responsible for transcriptionally activating ERE dependent expression in osteoblasts.

4.2. ER α shows high activity in the absence of ligand in 2T3 cells.

The activity activity of estrogen responsive reporters showed high activity in the absence of ligand. To determine if ER α could activate responsive promoters in the absence of ligand, a construct expressing mER α was co-transfected with ERE-tk-

luciferase reporter construct into subconfluent 2T3 cells. While sub-confluent, these cells express little ER α and consequently, induce little endogenous reporter gene activity (figure 14B). It is therefore possible to study a transfected ER α in this context without the contaminating activity of endogenous ER α.

Transfected mER α induced transcription of luciferase without addition of E2, to ~ 40% of the level obtained in the presence of saturating amounts of E2 (figure 15A). Furthermore, the human ER α receptor was also able to transactivate reporter gene expression in 2T3 cells, indicating that this phenomenon is not specific to the mouse receptor (figure 15A).

As the context and specific sequence of individual ERE's can greatly affect transactivation by estrogen receptors (Hall *et al.*, 2002), we wondered if activity observed in the absence of E2 was consequent on the reporter construct used. To evaluate this, a construct where luciferase expression was determined by the action of the pS2 promoter was used. The pS2 promoter is specifically responsive to estradiol bound ER (Masiakowski et al., 1982). Its activation by ER arises from an imperfect palindromic ERE, at position -393 respective to the major transcriptional start site, which differs from the ERE previously used at 2 bases (Berry et al., 1989).mER α achieved a similar level of ligand-independent activity on the pS2 promoter as on the ERE-tk-luciferase reporter indicating that the high constitutive activity of ER α is independent the promoter used and the ERE sequence (figure 15B).

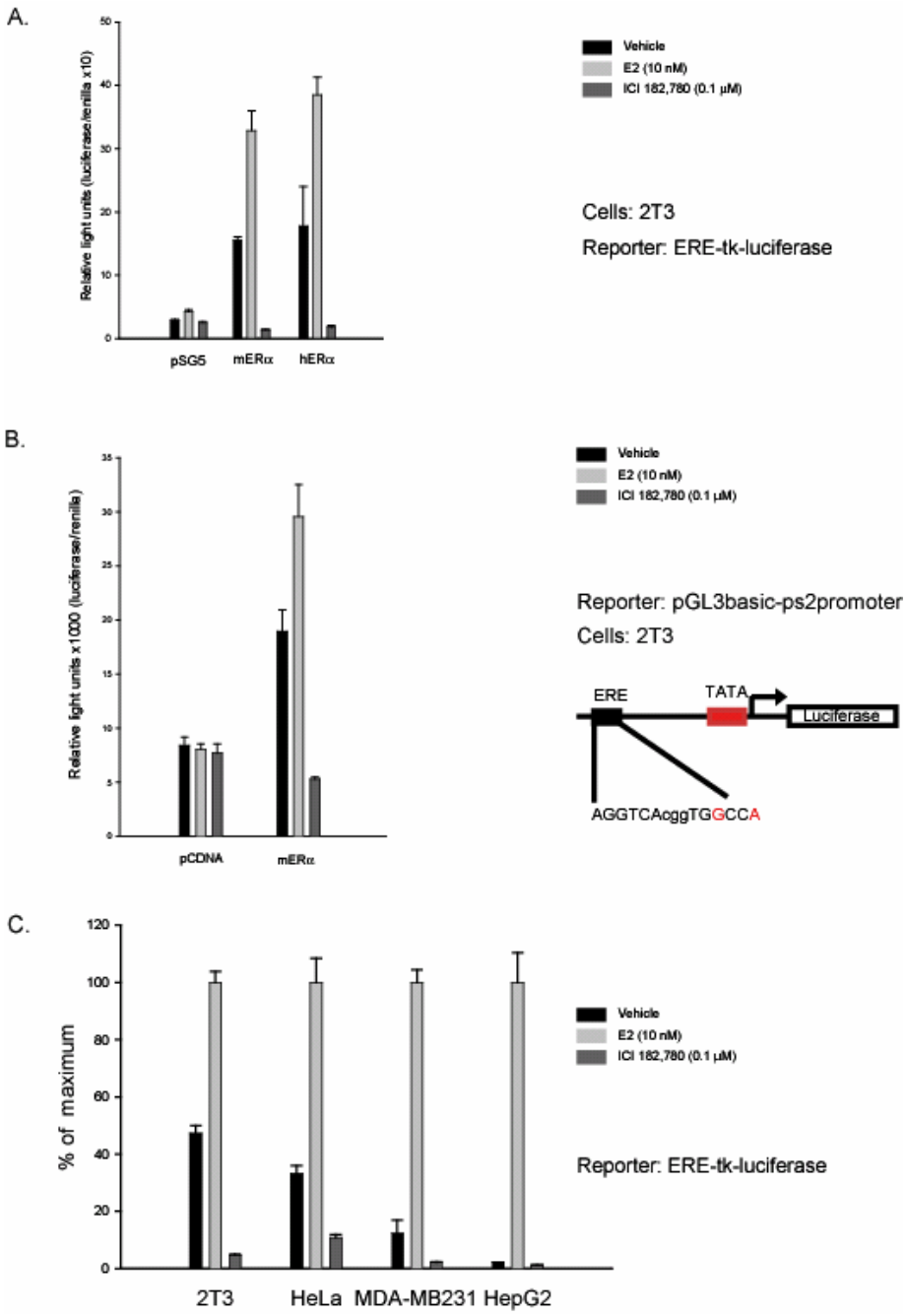


Figure 15. mER α constitutive activity is not promoter dependent but varies between cell lines.

A. Empty pSG5 vector as well as mER α, and hER α were transiently transfected in subconfluent 2T3 cells together with ERE-tk-luciferase reporter. Cells were treated 24h before harvest with vehicle, E2 or ICI 182,780. **B.** Subconfluent 2T3 cells were transiently transfected with the pGL3-pS2 promoter reporter construct and mER α or empty pSG5. Cells were treated as in A. **C.** Different cell lines were transiently transfected with mER α and the ERE-tk-luciferase. Results are plotted as % of maximum to show the variation in basal activity with vehicle alone.

Constitutive activity of ER has been observed in several contexts as upon growth factor treatment but rarely in untreated cells, at least not to such an extent. It was therefore of interest to determine if high constitutive activity could be obtained in cell lines in addition to the 2T3 line. Figure 15C depicts the percentage of ligand-independent activity obtained when several ER α negative cells lines were transfected with mER α. Constitutive activity ranges from ~ 40% in 2T3 cells to 2% in HepG2 cells. There is no apparent correlation between AF context and activity in the absence of ligand, as MDA-MB231 cells and 2T3 cells both support AF2 derived activity to similar levels (data not shown) but have yet very different reporter activities in the absence of ligand (47 and 12% respectively).

These data indicate that the constitutive estrogenic activity observed in osteoblasts is likely mediated by mER α. Moreover, ER α mediated constitutive activity varies between different cell contexts, however, within all cell types evaluated, it is highest in osteoblasts.

4.3. Molecular analysis of the "ligand-independent activity" of ER α

As outlined in the introduction, the ligand-independent activity of ER α has been extensively studied. In summary, the signal transduction pathways of several growth factors converge to activate ER α mediated signaling either directly by phosphorylation of the receptor or indirectly through phosphorylation of associated coactivators (Weigel and Zhang, 1998;Lopez et al., 2001;Dutertre and Smith, 2003b). Many of the phosphorylation events that achieve ligand-independent activity occur on serine residues located in the N-terminal part of the receptor (Dutertre and Smith, 2003a). In addition, a potential α-helix in the A domain was shown to preclude the active conformation of ER α in the absence of hormone, through occupying the

surface groove in the E domain that associates with the LXXLL motif of intermediate transcription factors (Metivier *et al.*, 2002b). To identify regions that have a role in the constitutive activity of ER α, a series of deletion mutants were constructed (figure 16). These constructs were then evaluated in transient transfection assays for their ability to induce estrogen dependent gene activation, using ERE-tk-luciferase as a reporter construct. As seen in figure 16, deletion of the A domain, although it was reported to increase ligand-independent activity (Metivier *et al.*, 2002b), did not affect activities either in the presence or absence of ligand. Likewise, deletion of both A and B domains had no effect on the activity of the receptor in the absence of hormone (figure 16). Deletion of the F domain also failed to affect the activity of the receptor both in the presence and absence of E2. These data show that the constitutive activity of ER α is unlikely to involve the A domain or phosphorylations in the B domain since a truncated receptor missing these domains still exhibits activity in the absence of ligand.

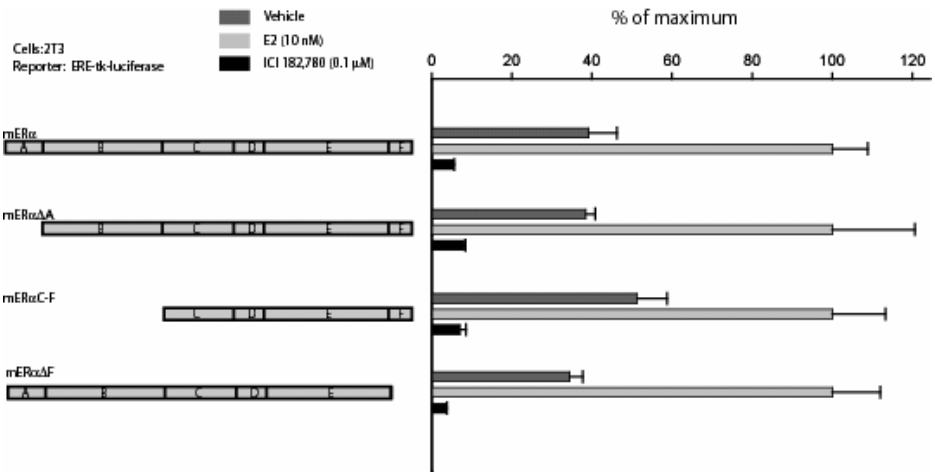


Figure 16. Ligand-independent activity of ER α truncation mutants

Several truncation mutants of mER α were transfected in subconfluent 2T3 cells and their transcriptional output was analyzed using the ERE-tk-luciferase reporter as previously described. The values are plotted as % of maximum.

4.4. The "ligand independent" activity of mER α arises from residual estrogens the culture medium

The constitutive activity of mER α may arise from estrogens remaining in the culture medium. These estrogens could come from the serum or from estrogenic contaminants present in the medium. Although the serum used was charcoal stripped, that is, estrogens and many other small, hydrophobic molecules removed by adsorption onto dextran-coated carbon, it is never completely devoid of steroids. It is possible that, in certain cell contexts, ER α is hypersensitive to little concentrations of contaminating estrogens.

To determine whether the high constitutive ER α activity observed in osteoblasts arises from estrogens present in the culture medium, we made use of a mutant ER α. Mutation of glycine 400, within the ligand binding domain, to valine (hERα G400V) decreases hER α affinity towards ligand (Tora et al., 1989a). Although binding affinity is decreased, hER α G400V transactivates responsive promoters to the same extent as hER WT at saturating hormone concentrations, indicating that coactivator binding is not directly affected by the mutation (Tora *et al.*, 1989a). Furthermore, this mutation was shown not to affect the induction of ligand-independent activity by EGF and forskoline/IBMX (Bunone *et al.*, 1996;el Tanani and Green, 1997). Taken together, hER α G400V is a good tool that distinguishes between hormone sensitivity and ligand-independent activity, in the analysis of the constitutive activity of ER α in osteoblasts.

As previously reported, mutation of Glycine 400 into valine did not affect induction of the reporter in the presence of saturating amounts of E2 (figure 17). However there was no induction of the reporter in the absence of hormone above the levels of ICI 182,780 treated cells. This result indicates that the apparent constitutive activity of ER

α observed in osteoblasts comes from residual estrogens present in the growth medium. The high residual activtity of ER α can result from higher estrogen levels in the culture medium of osteoblasts because they do not metabolize estrogens as efficiently as other cell types. Alternatively, the residual estrogen levels present in the growth medium might be the same as for other cell types, but ER α in osteoblasts is hypersensitive to estrogens and can respond to low estrogen concentrations.

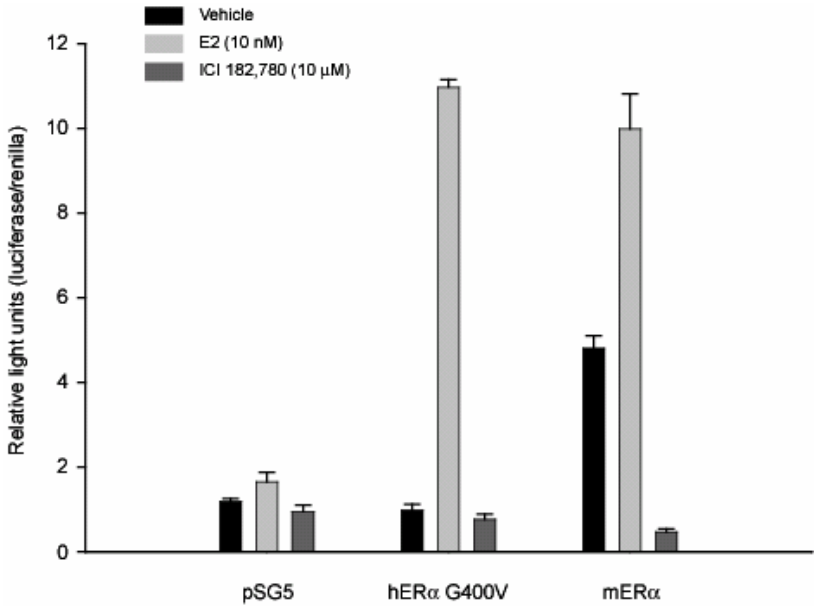


Figure 17. ER α G400V does not have any basal activity

Empty pSG5 vector, hER α G400V , and mER α were transiently transfected in subconfluent 2T3 cells and transcriptional activity was assessed on the reporter ERE-tk-luciferase after 24h treatment with vehicle, E2 or ICI 182,780.

5. Estrogen signalling is not necessary and does not affect differentiation of 2T3 cells

Because osteoblasts express a functional ER α, it is expected that estrogens can directly affect osteoblast differentiation or function. Several studies report a positive effect of estrogens on osteoblast differentiation while others show no effects (Keeting *et al.*, 1991;Scheven *et al.*, 1992;Qu *et al.*, 1998).

We therefore wanted to investigate whether 2T3 cells were able to react to continuous E2 treatment. Cells were kept in low estrogenic charcoal stripped medium and assayed for ALP activity every second day over a period of 8 days after confluency. The appearance of ALP positive cells was delayed in charcoal stripped medium as compared to cells grown in unstripped medium (compare figure 18A and 7B). However the continuous addition of 10 nM E2 did not modify the kinetics of ALP expression.

To determine whether estrogen signalling is necessary to osteoblastic differentiation, we also allowed 2T3 cells to differentiate in culture medium containing unstripped serum in the continuous presence 10 μM ICI 182,780. Figure 18B shows that there is no difference in the kinetics of ALP expression whether or not ICI 182,780 is added to the culture medium. We previously showed that ICI 182,780 is functional in inhibiting estrogen signalling in 2T3 cells as assessed by the transcription assays presented previously (figure 14B).

This assay would be able to detect possible effects of estrogens on proliferation, cell death, and differentiation alike. Modifying proliferation or cell survival will modify the rate of differentiation as differentiation is governed by cell contacts (Owen *et al.*, 1990;Aronow *et al.*, 1990). However, 2T3 cells are committed to the osteoblast

lineage and do not differentiate into other cell types in the absence of additional factors (Chen et al., 1998). It is therefore unlikely that the ALP assay can assess the possible ability of E2 to influence commitment of 2T3 cells to different mesenchymal cell lineages. Altogether these data indicate that estrogen signalling is not required for the process of osteoblast differentiation. Also, estrogens do not accelarate osteoblast differentiation at least as far as ALP expression is concerned.

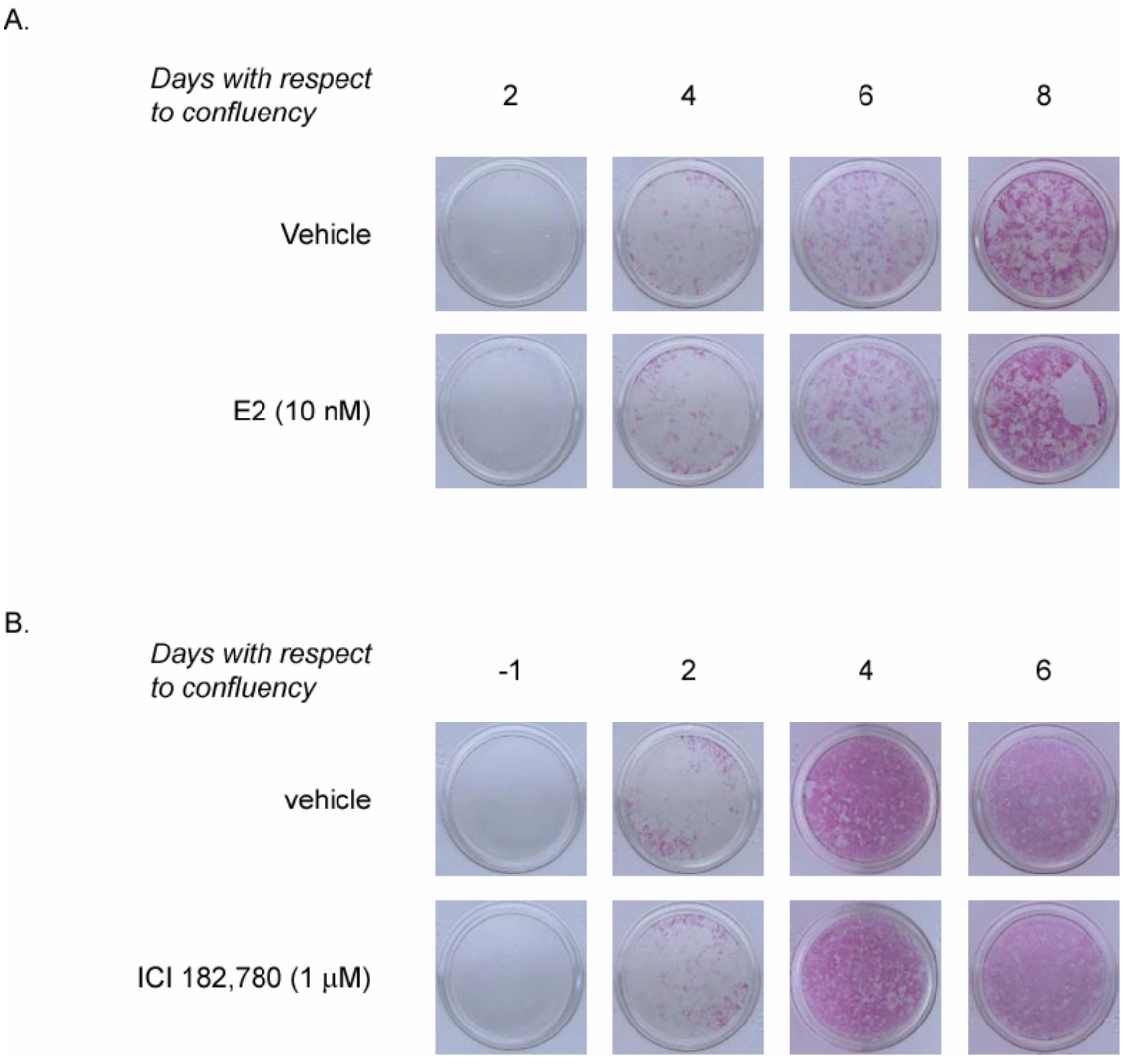


Figure 18. Estrogen signalling does not affect osteoblast differentiation

A. 2T3 cells were assessed for ALP expression over a period of 8 days postconfluency in charcoal stripped medium in the presence or absence of 10 nM E2. **B.** ALP expression of 2T3 cells grown up to 6 days postconfluency in normal medium containing vehicle or 1 μM ICI 182,780.

6. The effect of TGF beta on ER alpha expression and transactivation function

As we have seen, transient treatment with TGF β was able to downregulate mER α in 2T3 cells. We decided to investigate this phenomenon further. Interestingly, TGF β treatment was previously shown to affect ER α expression. Two different mechanisms have been described (Stoica *et al.*, 1997;Petrel *et al.*, 2003). One way would be through silencing of transcription from the A promoter of the ER α gene (Stoica *et al.*, 1997). The alternative mechanism involves increased proteasome mediated degradation of the receptor (Petrel *et al.*, 2003).

We initially determined if TGF β triggers proteasomal degradation of ER α. The results showed in figure 19A confirm as previously shown that degradation induced both by E2 and ICI 182,780 is proteasome mediated, as MG132, an inhibitor of proteasome activity, prevents these effects. TGF β also downregulates ER α, however, this downregulation is not accentuated when the cells are co-treated with E2 or ICI 182,780. Surprisingly, Treatment with TGF β and MG 132 resulted in a complete disappearance of ER α. Although the disappearance of mER α upon combined TGF β and MG 132 treatment cannot be explained, these results show that in 2T3 cells, TGF β mediated downregulation of mER α does not involve proteasome activity.

To find out whether TGF β induced downregulation correlated with decreased mRNA levels, total RNA was isolated from day 6 2T3 cells treated or not with TGF β. The result shows that treatment with TGF β decreases more than 30 folds the amount of ER α mRNA as compared to GAPDH mRNA (figure 19B).

Finally we determined if a decrease in mER α transcriptional activity ensues from the decrease in mER α levels following TGF β treatment. Accordingly, TGF β treatment

was able to diminish ER α mediated induction of the ERE-tk-luciferase reporter, both in the absence and presence of E2 and to levels below that of cells treated with ICI 182,780 (figure 19C).

In conclusion, downregulation of mER α induced by TGF β is not due to increased proteasome mediated degradation as MG 132 fails to block this process. Further confirmation that the proteasome is not involved in TGF β induced downregulation of ER α, is illustrated by the lack of synergism between TGF β and either E2 or ICI 182,780 in decreasing mER α levels. The TGF β-induced downregulation also occurs at the mRNA level and could involve transcriptional regulation of ER α expression as was previously suggested (Stoica *et al.*, 1997). Decrease in mER α protein levels was mirrored by a decline in transactivation activity.

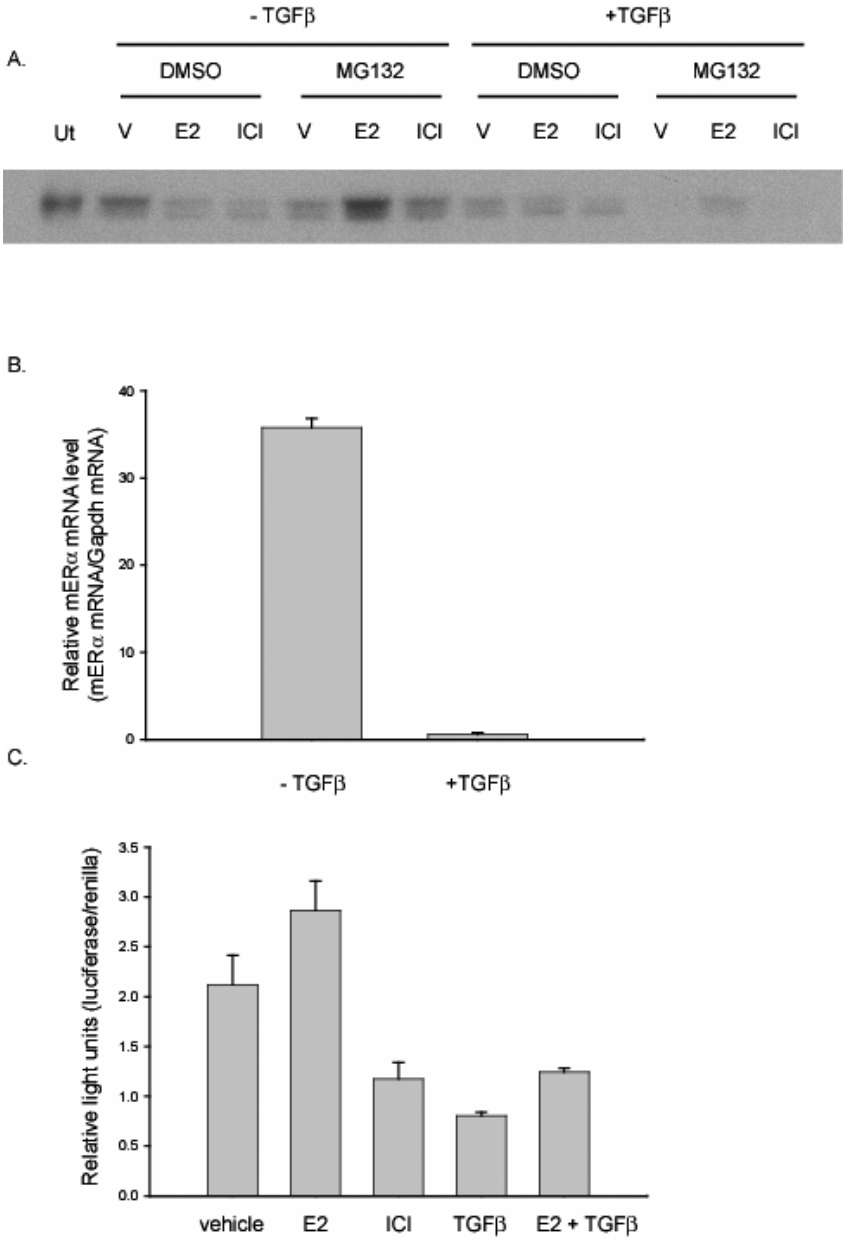


Figure 19. Decrease in mER α protein, mRNA and signalling after transient TGF β treatment.

A. Western blot analysis of endogenous mER α expression in 2T3 cells 6 days postconfluency with MC-20. Cells were treated concomitantly with ethanol vehicle, E2 (10 nM) or ICI 182,780 (0.1 μM) and DMSO or MG132 () and TGF β (5 ng/ml). 100 μg of protein was loaded per well. **B.** Total RNA was isolated from 2T3 cells 6 days after confluency treated or not with 5 ng/ml TGF β and subjected to quantitative RT-PCR analysis. The values obtained were normalized to GAPDH expression. **C.** 2T3 cells 6 days postconfluency were transfected with ERE-tk-luciferase reporter construct and treated for 24h with ethanol vehicle, E2, ICI 182,780 and TGF β (5ng/ml).

7. Human ER alpha referenced as wild type contains the mutation G400V

In the course of this study we analyzed the high constitutive activity of the mouse ER α in 2T3 cells and compared to the human receptor. We were initially using a mutated form of hER α thinking we were using WT receptor. The mutated hER α was mutated on glycine 400. The mutation G400V that we have described previously renders hER α less sensitive to ligand. As a result the ligand-independent activity seemed to be mouse specific as the mutated human receptor did not display any activity in the absence of E2. Unwittingly, as the reference sequence of wild type hER α cDNA available in databases corresponds to this mutated receptor, the G400V mutation was never identified after sequencing as being erroneous. This appendix describes the work done that led to the identification of this mutation.

As hER G400V was used, we tried to map the apparently mouse specific high-constitutive activity. To achieve this, a series of chimeras of mER α and hER α were constructed. Chimeras indicated that the E domain, encompassing residues 362 and 533 of mER α and residues 357 and 537 of hER α, were sufficient to confer or abrogate constitutive activity in hER α and mER α respectively (figure 20).

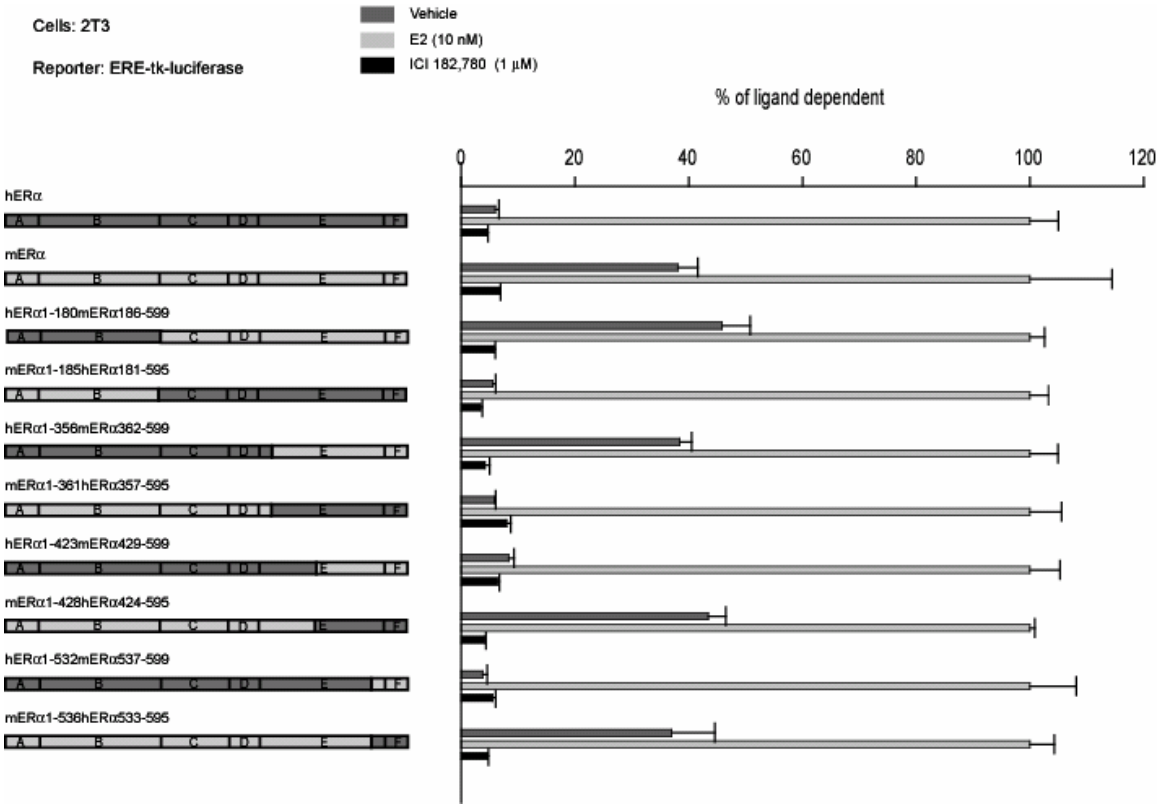


Figure 20. The apparent ligand-independent activity of mER α is localized to the E domain (amino acids 362-533).

Chimeras were constructed between hER α and mER α as depicted on the left. hER α mER α and the multiple chimeras were transfected in subconfluent 2T3 cells and luciferase expression assessed from the ERE-tk-luciferase reporter after 24h vehicle, E2 or ICI 182,780 treatment. The results are plotted as % of maximum.

Comparison of the protein sequences in the region responsible for the different constitutive activities, revealed two divergent amino acids between the mouse and the human receptors that were responsible for the constitutive activity of the human and mouse ER α (figure 21A). Single and compound mutations of these amino acids to their human or mouse counterparts were then generated. None of the mutations either single or compound were able to change the activity of the receptor in the absence of ligand.

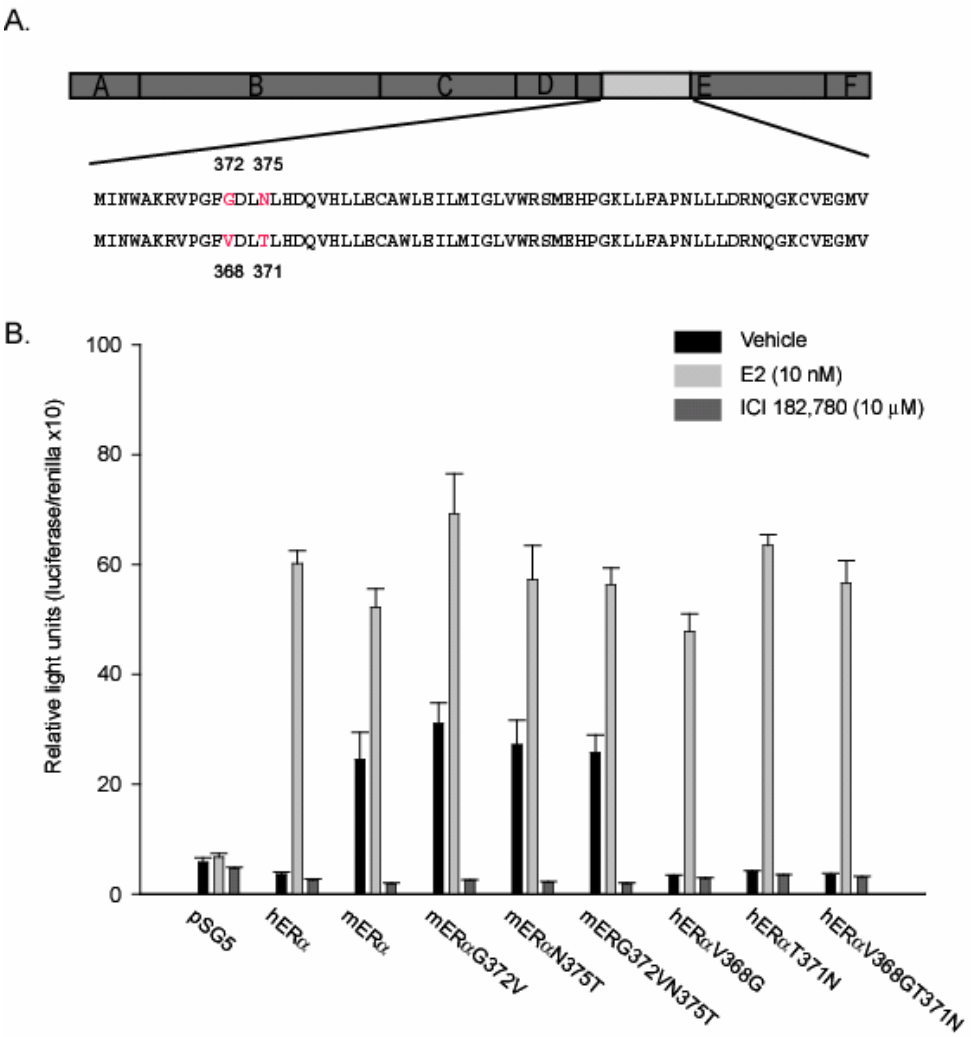


Figure 21. Mutation of the divergent amino acids between hER α and mER α to their mouse and human counterparts does not affect ligand-independent activity.

A. Alignment of hER α and mER α of the region defined as necessary and sufficient for ligand-indepdent activity showing the two divergent amino acids in red. B. Analysis of single of compound mutations of the two divergent amino acids. The different mutants were transfected in subconfluent 2T3 cells and transactivation assessed on the ERE-tk-luciferase after treatment with vehicle, E2 and ICI 182,780 as previously described.

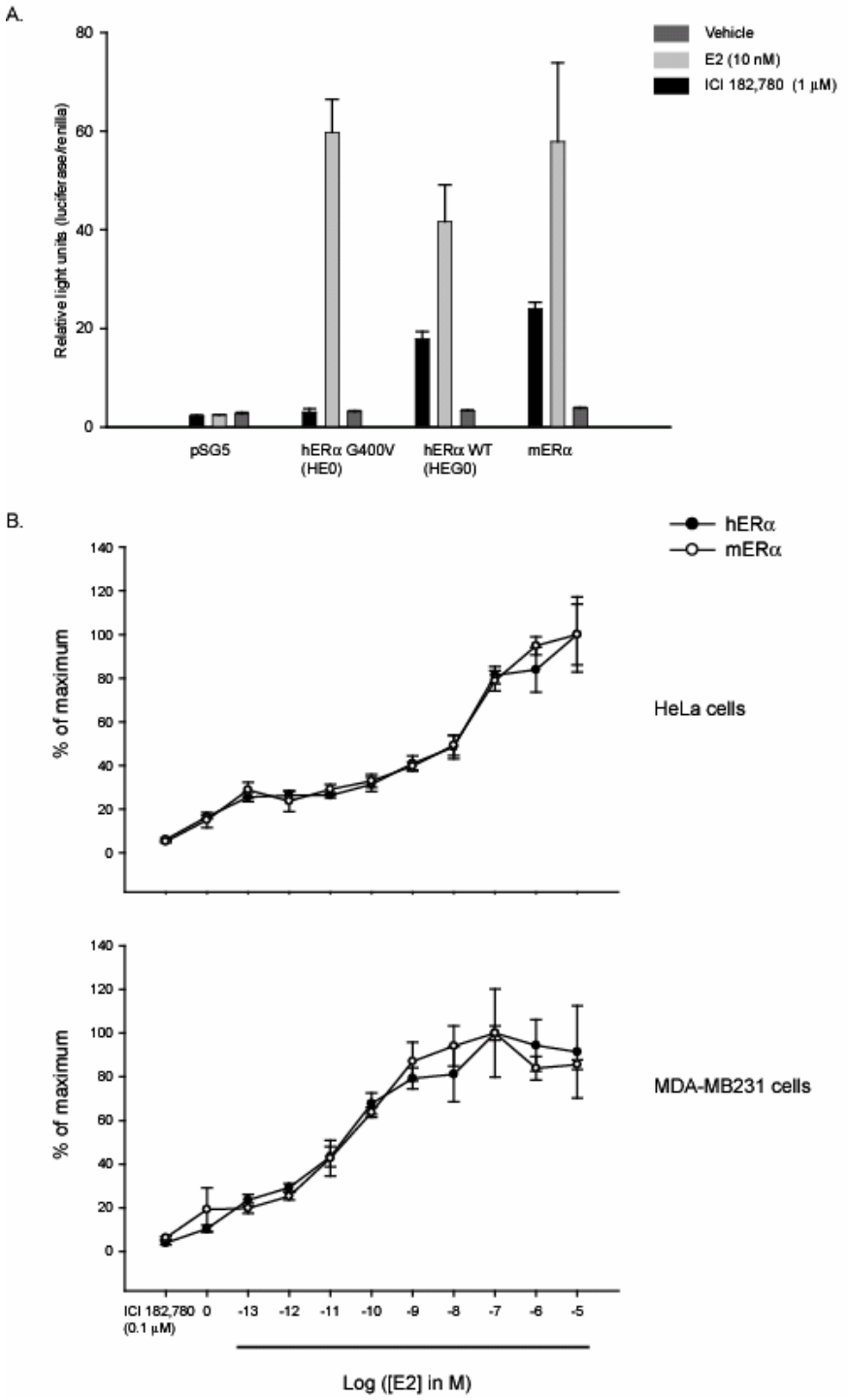


Figure 22. Mouse and human ER α have the same responsivity to E2.

A. Empty pSG5, hER α G400V, hER α WT and mER α were transfected in subconfluent 2T3 cells and luciferase activity from the reporter ERE-tk-luciferase assayed. **B.** Dose response assay of hER α and mER α in MDA-231 and Hela cells. Cells were transfected with WT hER α and mER α and increasing concentration of E2 were used. ICI 182,780 was used to determine basal level. The values are plotted as % of maximum.

Since mutations of mER α or hER α were not able to revert either of the phenotypes, it was hypothesized that the constructs used could have been mutated on other residues within the region mapped. Every construct used was sequence validated and found to be correct; the only possibility left was therefore that database reference sequences were incorrect. Alignment of the translated cDNA sequences used (Reference number NM_000125) to validate our constructs indeed revealed a divergence at residue 400 of hER α (residue 404 of mER α). This divergence does not appear when directly aligning the protein sequences available.

The sequence of hER α corresponds to the original isolate that was subsequently shown to be mutated at position 400 from glycine to valine. This mutation was shown to affect affinity to the ligand and consequently sensitivity to E2 (Tora *et al.*, 1989a). Using a non-mutated hER α cDNA showed that human and mouse receptors had the same activity in the absence of estradiol in 2T3 cells and the same sensitivity to estradiol in MDA-MB231 and HeLa cells (figure 22A and B).

Discussion

1. ER alpha is expressed and is functional in mouse osteoblasts

Osteoblasts are believed to be the direct target of estrogens since the discovery of a small number of E2 binding sites (Komm *et al.*, 1988;Eriksen *et al.*, 1988). The expression of ER α has been extensively studied at the mRNA level; however detection of the protein is difficult because of the low expression levels. Nevertheless, it is assumed that, despite the low amounts of ER α present, osteoblasts mediate some if not all of the effects of estrogens on bone (Spelsberg et al., 1999;Riggs *et al.*, 2002;Manolagas et al., 2002). It is still controversial however as to whether the amounts present are sufficient to respond to estrogens (Karsenty, 1999).

Using a well-characterized *in vitro* differentiation system, we describe here the expression of ER α in murine osteoblasts. This work demonstrates that two protein products with the biochemical characteristics of ER α are expressed both in primary osteoblasts and in the osteoblastic 2T3 cell line (figure 8,).

This endogenous ER α could specifically transactivate an ERE-tk-luciferase reporter construct, demonstrating that functional signalling occurs in these cell types (figure 14). There are good indications that the endogenous activity detected in osteoblasts results from the presence of ER α. Indeed, there is an excellent correlation between ER α expression and inducibility of the reporter. Firstly, the activity in osteblasts increases with differentiation concomitantly with ER α expression (figure 14). Moreover, downregulation of ER α expression by TGF β induces a decrease in overall reproter activity (figure 19). The data presented does not absolutely rule out that ER β is involved in the transcriptional activity observed. When ER β was transfected in 2T3 cells, it displayed the same residual activity as ER α in the absence of additional E2.

Therefore, ligand-independency could not be used to discriminate between ER α and ER β (data not shown). ER β expression is however low in osteoblasts and does not vary with differentiation and would therefore not correlate with the activity of the ERE-tk-luciferase reporter (Zhou et al., 2001;Wiren *et al.*, 2002).

On the other hand, the orphan nuclear receptor ERR α has been detected in osteoblasts and shown to be active (Bonnelye *et al.*, 2001). ERRs, like ERs recognize EREs, but do not bind ligands and have constitutive activity (Vanacker *et al.*, 1999). Interestingly, endogenous transcriptional activity detected in osteoblasts was also mainly constitutive. However, ERRs do not to respond pure antagonists of estrogen, like ICI 182,780 (Vanacker *et al.*, 1999). However, ICI 182,780 decreased the transcriptional activity observed in osteoblasts, indicating that an ER is responsible for transactivation of ERE dependent promoters.

Altogether, the data presented here prove that ER α is expressed and transcriptionally active in murine osteoblasts.

2. ER alpha exists as two isoforms in mouse osteoblasts

The ER α gene is a complex genetic unit consisting of several promoters and multiple exons. Alternative promoter usage and alternative splicing generates different transcripts, some of which giving rise to shorter isoforms. The best documented isoform of ER α is ER α 46, which has been reported to result either from the splicing of one of the upstream exons to exon 2, or from internal ribosome entry (Barraille *et al.*, 1999;Flouriot *et al.*, 2000). The ER α 46 protein isoform has been detected in the human breast carcinoma cell line MCF7 and in human primary osteoblasts (Flouriot *et al.*, 2000;Denger *et al.*, 2001). In human osteoblasts, ER α 46 is expressed at a similar

level to the full-length protein (Denger *et al.*, 2001). ER α 46 lacks the A and B domains including the entire AF1. ER α 46 can still dimerize and bind to DNA. ER α 46 can dimerize with the full-length receptor and inhibit its transcriptional activity as a result of the AF-1 supporting context found in human osteoblasts (Denger *et al.*, 2001). In addition, the transcript resulting from the splicing of upstream exon F to exon 2 that would code for the ER α 46 was identified in mouse bone (Denger *et al.*, 2001). Together, these data suggest that ER α 46 could play a major role in the response of osteoblasts to estrogens.

Here, no evidence of a 46 KDa ER α isoform in murine cells was found, either in uterus or in osteoblasts although the corresponding transcripts were detected (figure 8 and data not shown). It could be that in mouse osteoblasts the amounts of corresponding transcript are so low compared to full-length transcript that the corresponding protein cannot be detected. Alternatively, the two initiating AUGs in exon 2, which are also present in the mouse ER apha, might not be effective at initiating translation. These results, however, rule out a possible involvement of ER α 46 in the regulation of osteoblast physiology in the mouse.

This notwithstanding, mER α runs as a doublet on SDS-PAGE, suggesting that it exists as two molecular entities of similar molecular weight. A comparable doublet was also detected on a western blot of the endogenous mER α from mouse ES cells, using the same antibody as in this study, but was not commented upon (Perissi et al., 2004). The doublet is not present in preparations from uterus but this may be a consequence of the larger amounts of mER α present in this tissue. Both bands are degraded in response to E2 or ICI 182,780 confirming that the two products are mER α (figure 8A). The nature of the two products can only be speculated upon. ER α was previously shown to migrate as a doublet on SDS-PAGE (Golding and Korach, 1988).

This was found to result, at least partly, from phosphorylation of ER α on serine 118 induced by E2 treatment (Joel et al., 1995;Joel *et al.*, 1998). It is however improbable that the dimorphism of mER α results from phosphorylation of serine 122 (the mouse ortholog of serine 118) as E2 treatment that would promote its phosphrylation, does not affect specifically one of the two bands (figure 8A). Moreover, exogenously expressed HA-tagged construct does not display dimorphism further indicating that it is unlikely that phosphorylation is responsible for the appearance of the upper band (figure 8C). The absence of the doublet with the HA-tagged mER α also rules out any other posttranslational modification as the cause of dimorphism.

Because an N-terminal antibody recognizes both bands but only one is recognized by a C-terminal antibody, it is plausible that the lower band corresponds to an N-terminally deleted form of ER α (figure 8C). No ER α mRNA variant has been identified to date that could account for an N-terminally-truncated isoform of this size. Interestingly, a similar doublet has never been observed in tissues or cells of human origin (unpublished observations). This mouse specific effect may be explained by the presence of two ATGs in exon 1 that occur in mouse but not in human 87 base pairs downstream of the AUGs used to produce full-length ER α. The use of these AUGs would give rise to a protein of 63.5 KDa, as opposed to the 67 KDa predicted for the mouse full-length ER α. This size difference is consistent with what is seen on a western blot. There is evidence that translation by ribosome scanning occurs with the human and mouse ER α transcripts (Kos *et al.*, 2002). As a result, leaky scanning of the initating AUGs would allow translation to start from the downstream AUGs in exon 1. It may be argued that the lower band should occur on expression of the HA-tagged ER αs, however theoretical considerations indicate that this is unlikely. Since the initiating AUG in the HA-tagged constructs is within a

strong consensus Kozak sequence and, if translation initiation occurs by ribosome scanning, the strong initiating AUG will prevent alternative scanning and translation initiation from the downstream AUGs thereby occluding translational initiation on alternative AUG's. Interestingly, the truncated receptor would be devoid of A domain and would be predicted to have high levels of activity in the absence of ligand in AF-1 promoting contexts while it would repress transcription in AF-2 permissive contexts (Metivier et al., 2002c). This shorter product however would not be responsible for the high basal transcriptional activity of ER α in osteoblasts, as deletion of the A domain of ectopically expressed ER α does not alter its activity in 2T3 cells (figure 16). Although this assumption is very speculative it merits further investigation.

3. ER alpha expression in the mesenchymal lineage is not specific to osteoblasts and is lower than in reproductive tissues.

Osteoblasts arise from pluripotent mesenchymal cells. These cells differentiate not only into osteoblasts but also into myocytes, adipocytes and chondrocytes given appropriate extracellular cues. Mesenchymal stem cells differentiate in accordance with the multi-lineage priming model (Hu et al., 1997). This model states that pluri-potent progenitor cells express low level of genes specific to all the lineages they can differentiate into. Upon differentiation into a given cell type, genes corresponding to other lineages are repressed, while expression of lineage specific genes increases. This model applies to osteoblast progenitors which express low levels of myogenic and adipogenic markers prior to the onset of osteoblastic differentiation (Garcia *et al.*, 2002). The inception of osteoblast differentiation corresponds to activation of the

osteoblast specific transcription factor Cbfa1 (Ducy *et al.*, 1997). Upon activation by increased expression and by phosphorylation, Cbfa1 triggers an osteoblast-specific genetic programme, which results in the sequential expression of osteoblast-specific transcription factors and proteins involved in osteoblast function, such as matrix synthesis and mineralization (Ducy *et al.*, 1997;Lee *et al.*, 2000;Alliston *et al.*, 2001).

There are some indications that ER α could be involved in an osteoblast specific genetic programme and participate in the regulation of osteoblast differentiation. First, there are several reports showing that ER α expression is amplified with osteoblast differentiation both in human and rat (Komm *et al.*, 1988;Arts *et al.*, 1997;Wiren *et al.*, 2002). This indicated that ER α is up-regulated by osteoblast differentiation signals and integrated in the osteoblast-specific genetic programme. Moreover, transfected ER α interacts with Cbfa1 to enhance its activity in osteoblasts, confirming the integration of ER α in osteoblast differentiation (McCarthy *et al.*, 2003). On the other hand, ER α expression is relatively low in osteoblasts and ER α is ubiquitously expressed at a low level which argues against a specificity of ER α expression in osteoblasts (Eriksen *et al.*, 1988;Davis *et al.*, 1994;Couse *et al.*, 1997;Karsenty, 1999).

According to the multi-lineage priming model, we expected ER α to be expressed at a low level in undifferentiated osteoblasts and its expression to increase with differentiation. Conversely, blocking differentiation or differentiation into another cell type should result in the suppression of ER α expression.

In accordance with the studies mentioned above, we found that ER α expression increased concomitantly with osteoblast differentiation both at the protein and mRNA level (figure 11A and 11B). This increase was mirrored by an increase in the activation of an estrogen responsive ERE-tk-luciferase reporter construct (figure 14).

TGF β can provide competence for early stages of osteoblastic differentiation, but it inhibits myogenesis, adipogenesis, and late-stage osteoblast differentiation. Although it promotes the expression of collagen type I, it prevents the expression of osteoblast markers such as ALP and osteocalcin (Centrella *et al.*, 1994;Alliston *et al.*, 2001;Spinella-Jaegle et al., 2001). In preventing osteoblast differentiation, it functionally blocks activation of Cbfa1 on certain promoters, thereby stopping differentiation from proceeding (Alliston *et al.*, 2001). Using 2T3 cells, we found that, continuous TGF β treatment blocks differentiation of 2T3 cells in an ALP negative stage without affecting cell proliferation (figure 12A and 12B). We therefore used this model system to investigate whether ER α expression was linked to osteoblast differentiation and if TGF β treatment prevented its up-regulation. TGF β was able to down-regulate ER α expression but only when cells were treated for 24h and not after long-term (6 days) continuous treatment. The effect of transient TGF β treatment on ER α expression will be discussed later, as it is not related to differentiation. We found however that ER α expression increased whether or not the cells had been treated continuously with TGF β, that is, independently of ALP expression (figure 12C). This result suggests that ER α expression does not correlate with osteoblast differentiation. It was however still possible that ER α expression increased with the early stages of osteoblastic differentiation.

To further examine the premise that ER α expression is not tied to osteoblast differentiation, we used the pluripotent C2C12 cell line that differentiates with confluency into myoblasts but can be efficiently induced to differentiate into ALP positive osteoblasts with high doses of BMP2 (Katagiri *et al.*, 1994). Like TGF β, BMP2 down-regulated ER α protein levels after transient treatment. This phenomenon will be discussed later together with the effects of transient TGF β treatment on ER α

expression. The expression of ER α increased whether cells progressed to multinucleated myotubes or differentiated into ALP positive osteoblasts (figure 13B). There was moreover no difference in ER α expression between myoblast-differentiated C2C12 cells and osteoblast-differentiated C2C12 cells at day 6 with respect to confluency (figure 13B). The increase in ER α levels during osteoblast differentiation is not a direct consequence of osteoblast differentiation since it also occurs during myoblastic differentiation. It is interesting to note that increase in ER α expression is not a specificity of the mesenchymal lineage as an increase in ER α levels has also been detected during the differentiation of the mammary epithelial cell line HC-11 and during the maturation of B cells (Grimaldi et al., 2002;Faulds et al., 2004). It is not known whether a common mechanism is responsible for the upregulation of ER α expression in these unrelated cell types.

Although osteoblasts contain more ER than fibroblasts (Eriksen *et al.*, 1988), they nevertheless contain at least a thousand fold less than an estrogen target tissue like uterus (Davis *et al.*, 1994). We confirmed these results in mouse by directly comparing the amounts of ER α protein in mouse primary osteoblasts and in uterus (figure 10). As we found here, the level of expression of ER α between an unambiguous target tissue like uterus and osteoblasts or myoblasts is indeed considerable.

These results tally with the concept that ER α is expressed ubiquitously in an unspecific way at a low level. As mentioned in the introduction ER α has been detected in numerous if not all tissues and cell types examined so far. On the other hand, in specific target tissues like the reproductive tract high levels of ER α are present.

What could direct the ubiquitous low level expression of ER α? As detailed in the introduction, the ER alpha mRNA can be transcribed from at least 7 different promoters. Interestingly, in mouse the F promoter seems to be expressed at a similar level in every tissue examined included in (Kos *et al.*, 2000). In contrast, the C variant although it is detectable in many tissues, constitutes the main mRNA variant in uterus (Kos *et al.*, 2000). Consequently, it is plausible that the F promoter is responsible for ubiquitous ER α expression, while the C promoter directs the high level expression of ER α in tissues like uterus. It will be very informative to determine quantitatively the variants expressed in mouse osteoblasts. However, experimental data is still missing concerning the determinants of tissue-specific ER α expression.

Independent of the fact that ER α expression in osteoblasts is unspecific, the question remains: Can the low levels of ER α in osteoblasts mediate a response to estrogens?

4. Involvement of estrogen signalling in osteoblast differentiation

Estrogens have two different effects on bone: The osteogenic effect in which estrogens affect osteoblast number and function resulting in increased bone mass and the osteoprotective effect in which estrogens counteract the increase in osteclast and osteoblast number and function following estrogen deprivation. We wanted to investigate whether these two effects could be directly mediated by osteoblasts.

It is well accepted that estrogens can *in vivo* induce *de novo* bone synthesis (Samuels *et al.*, 1999). This action is largely a consequence of increased osteoblastic cellular number and activity (Samuels *et al.*, 1999). This so-called osteogenic effect of

estrogens is entirely dependent on ER α both in males and females (McDougall *et al.*, 2002;McDougall *et al.*, 2003). *In vitro* however, conflicting results have been reported. It was found that estrogens could enhance the number and activity of purified mouse bone marrow osteoblasts however others did not monitor any changes (Keeting *et al.*, 1991;Qu *et al.*, 1998;Qu et al., 1999). Because the little effect estrogens can have on osteoblasts *in vitro* does not mirror the effects estrogens have on osteoblast *in vivo*, it is still disputed whether the osteogenic action of estrogens is the consequence of a direct effect on osteoblasts (Takano-Yamamoto *et al.*, 1990).

Having found that 2T3 cells and primary osteoblasts contained ER α that could transactivate, we wanted to examine whether estrogens could affect osteoblast differentiation. Using 2T3 cells and monitoring ALP activity over time, we were not able to see any effect of E2 on osteoblast differentiation (figure 18A).

It is possible that the presence of residual estrogens in our system at near saturating concentrations for ER α as detected in the transcription assays would impair the detection of effects additional estrogens could have. However, if this were the case, the antagonist ICI 182,780 should have been able to counteract the effects of residual estrogens and consequently to inhibit differentiation, which was not the case (figure 18B). It could be argued that estrogens affect osteoblasts at steps distinct from ALP expression. Estrogens can indeed increase osteoblast function by increasing cell number, modulating cell survival, and proliferation (Qu *et al.*, 1998;Damien et al., 2000;Zhou *et al.*, 2001). Because cell number is crucial to the onset of differentiation, this assay should, however, be able to monitor defects upstream of differentiation, like proliferation and cell death (Aronow *et al.*, 1990). Alternatively, it is possible that estrogens influence osteoblast differentiation at an earlier commitment step that cannot be appreciated using 2T3 cells. It has been proposed that estrogens promote

osteoblast differentiation as opposed to adipocyte differentiation of mesenchymal progenitor cells in the bone marrow, hence increasing the number the number of osteoblasts (Dang *et al.*, 2002;Okazaki *et al.*, 2002).

As mentioned earlier, this result is however not entirely surprising, as estrogens were shown in many instances not to affect osteoblasts *in vitro* (Canalis and Raisz, 1978;Keeting *et al.*, 1991;Spelsberg *et al.*, 1999). This raises the question as to whether osteoblasts are the direct mediators of estrogen action during the osteogenic response. In rat, the osteogenic effects of estrogens were reported to be direct without central relays as local estrogen application affects bone density in one limb without affecting the contralateral one (Takano-Yamamoto *et al.*, 1990). It is therefore possible that another cell type in the bone environment signals to the osteoblasts upon estrogen treatment. It is however noteworthy that the osteogenic response to estrogens in rat requires much lower concentrations than in mice and could therefore involve an entirely different mechanism (Turner, 1999).

Our study confirms however that estrogen signalling is not necessary to osteoblast differentiation. Indeed growth of cells in the presence of the pure antiestrogen ICI 182,780 did not affect ALP expression in 2T3 cells (figure 18B). This supports the observation that ER α and ER β deficient primary mouse osteoblasts develop normally *ex vivo* (S. Dupont, personal communication). Decreasing estrogen levels by gonadectomy, or ablation of their two receptors results in an increase in osteoblast and osteoclast numbers and activities with osteoclast resorption overpowering bone deposition, eventually leading to bone loss (Oz *et al.*, 2000;Sims *et al.*, 2002;Sims *et al.*, 2003). Addition of estrogens can in this low estrogen context reverse bone loss, decreasing both osteoblast and osteoclast activities .The osteoprotective effect is essentially mediated by ER α, ER β playing a small redundant role in female (Sims *et*

al., 2003). If this osteoprotective effect on osteoblasts were direct, one would expect that inhibition of estrogen signalling would result in an increase in osteoblast differentiation. It is therefore probable that the increase in osteoblast number and function arising from deficient estrogen signalling is not directly mediated by osteoblasts.

Since the discovery of estrogen receptors in osteoblasts and osteoclasts it has been taken for granted that estrogens affect bone metabolism in a direct way. However estrogens can have pleiotropic effects and affect homeostasis by regulating the expression of other hormones and not necessarily in a direct way. Estrogens act on the pituitary, which secretes numerous hormones. Estrogens play a crucial role in regulating the levels of other hormones that are known to affect bone metabolism in a direct manner. Examples are GH (Leung et al., 2004) and Prolactin (Clement-Lacroix et al., 1999). There is now also good evidence that the endocrine control of metabolism and reproduction are interconnected (Takeda et al., 2003). Even if some of the effects of estrogens on bone are direct, indirect effects mediated by other hormones are bound to play a crucial role. Cell-type specific knockouts would help characterizing the place and mechanism of action of estrogens on bone.

5. Activity of ER alpha in osteoblasts

The ligand-independent activity of steroid receptors has been an issue for more than 10 years, since the discovery that certain growth factors and hormones could activate receptors without addition of ligand (Power et al., 1991;Smith *et al.*, 1993). This phenomenon was also found to occur in vivo as growth factors elicit an estrogenic response in the uterus requiring ER α (Ignar-Trowbridge et al., 1992;Curtis et al.,

1996). Ligand-independent activity would involve the phosphorylation of serine residues in the B domain leading to ligand-independent recruitment of coactivators (Lannigan, 2003;Dutertre *et al.*, 2003c). Interaction of the A and E domains of ER α is also important in suppressing activity of the receptor in the absence of ligand (Metivier *et al.*, 2002b). Importantly, any event that triggers activity in the absence of ligand must directly or indirectly disrupt the interaction between the A and E domains to allow coactivator recruitment.

Interestingly, the activity of ER α has high levels of activity even in the absence of estradiol both in primary osteoblasts and 2T3 cells (figure 14). This high constitutive activity was also present when ER α was transfected into undifferentiated 2T3 cells albeit to a lesser extent (figure15A). The high basal activity of ER is unlikely to be a reporter-related or transfection-related artefact. Firstly, a transiently transfected reporter and a stably integrated transgene showed the same high residual activity with the endogenous ER α (figure 14). Furthermore, the use of a different promoter upstream of the luciferase reporter did not influence this seemingly ligand-independent activity (figure 15B).

This high basal activity was neither altered by the deletion of domains A, B or F (figure 16). This result rules out any involvement of the serines located in the B domain in the constitutive activity. It also dismisses a possible role of the A domain in the ligand-independent activity observed in osteoblasts.

The ligand-independent activity varied however greatly between different cell lines, being highest in osteoblasts and lowest in the hepatic cell line HepG2 (figure 15). Interestingly, HepG2 cells because of their liver origin possess the enzyme necessary to metabolise estrogens (Mattick *et al.*, 1997). It was therefore possible that ER α in HepG2 cells does not display any ligand-independent activity because estrogens

present in the culture medium are efficiently metabolised, and that the high activity observed in the absence of ligand in osteoblasts is the result of residual estrogens.

To examine the possibility that residual estrogens are responsible for the high constitutive activity in osteoblasts, we used a mutant ER α that is less sensitive to estrogens. The G400V mutant ER α, although it does not transactivate in the presence of low estrogen concentrations, can however be activated to the same extent as WT ER α in the presence of saturating E2 concentrations (Tora *et al.*, 1989a). Importantly, the G400V mutant receptor is still activated by growth factors in a ligand-independent manner, indicating that this function is still active in the mutant receptor (Bunone *et al.*, 1996). If the high basal activity of ER α in osteoblasts is due to residual estrogens the G400V mutant should not be activated by the low concentrations present in the culture medium. ER α G400V did not show any activity as opposed to WT in the absence of additional E2 strongly indicating that the basal activity is the result of estrogens present in the growth medium.

The fact that osteoblasts specifically support such a high basal activity however does not necessarily mean that they do not metabolise estrogens and that there is as a result a higher estrogen concentration in the culture medium. It is also possible that ER α in this cell type is highly sensitive to estrogens and reacts to the low estrogen concentrations present in the medium.

The sensitivity of nuclear receptors can be for instance modulated by coactivator and corepressor levels, which vary between different cell types (Herdick and Carlberg, 2000). The number of receptors per cell is also an important factor and could explain why endogenous ER α shows higher basal activity than transfected ER α (Webb *et al.*, 1992).

Interestingly, using transgenic mice containing a estrogen responsive promoter it was found that non-reproductive tissues like brain and bone displayed high levels of luciferase reporter activity (Ciana et al., 2003). The basal activity observed was decreased after treating the mice with ICI 182,780. The authors concluded that ERs could function in a ligand-independent manner. Our results indicate however, that this high activity could be due to the high sensitivity of ER alpha or to higher local estrogen concentrations in these tissues.

6. Effect of TGF beta family members on ER alpha expression

The TGF β family of cytokines controls a diverse set of cellular processes. They exert potent effects on bone metabolism and directly affect osteoblast differentiation. While using TGF β to inhibit osteoblast differentiation, we found that a 24 h treatment with TGF β of 2T3 cells, whether differentiated or not induced a decrease in ER α protein levels. TGF β and estrogen signalling have long been known to interact (Knabbe et al., 1987). Two different mechanisms for down-regulation of ER α by TGF β in human breast carcinoma cell lines have been proposed. Firstly, ER α A promoter activity is reduced by TGF β in the human MCF7 cell line (Stoica *et al.*, 1997). Secondly, using several different human breast carcinoma cell lines, it was shown that TGF β induced proteasome-dependent ER α downregulation by TGF β was not at the transcriptional level but involved proteasome-dependent degradation of the receptor (Petrel *et al.*, 2003). In 2T3 cells, downregulation invoked by TGF β correlated with a decrease in ER α mRNA levels. In our hands, blocking proteasome activity did not prevent downregulation of the receptor but instead intensified the decrease in

intracellular level of ER α. Also, TGF β mediated downregulation of ER α protein levels resulted in a decrease in ER α transcriptional activity.

Although it has not been investigated further, a single 24h treatment of C2C12 cells with BMP 2 also decreased ER α protein amounts, independent of the differentiation status of the cells. It is appealing to postulate that downregulation of ER α by BMP2 involves a mechanism similar to that of TGF β. TGF β and BMP2 belong to the same cytokine family and upon binding to their receptors activate common intracellular effectors (Shi *et al.*, 2003). Interestingly, although they have antagonistic effects on osteoblasts, BMP2 and TGF β both up-regulate Cbfa1 expression in C2C12 cells (Lee *et al.*, 2000;Spinella-Jaegle *et al.*, 2001;Lee *et al.*, 2002). It is therefore possible that TGF β and BMP2 regulate ER α expression in osteoblasts through a common mechanism. It would be interesting to determine if this observation can be extended to other cell types in which ER α expression is pathological and to further characterize the mechanism(s) involved in ER α down-regulation.

Chronic TGF β and BMP-2 treatments did not however affect ER α expression. Although it has never been reported in osteoblasts, it is known from other cell types that activation of the TGF β receptors results in ligand-mediated receptor inactivation. This is achieved either through proteasomal degradation of the receptor or of its downstream effectors (Shi *et al.*, 2003). In 2T3 cells or C2C12 cells, chronic treatment with TGF β or BMP 2 would inactivate cognate signalling and as a result down-regulation of ER α would no longer occur.

7. Human ER alpha referenced as wild type contains the G400V mutation

The human ER α cDNA was isolated from a cDNA library of the human breast carcinoma cell line MCF7 (Walter et al., 1985). In 1986, the sequence of the newly cloned human ER α cDNA was published by two different laboratories (Green *et al.*, 1986;Greene *et al.*, 1986). Three years later however, it was reported that the original cDNA cloned contained the mutation G400V that renders the receptor less sensitive to estrogens (Tora *et al.*, 1989a). Since then however, the most cited cDNA sequence for hER α still corresponds to the mutated receptor hER α G400V that was initially cloned (Green *et al.*, 1986;Tora *et al.*, 1989a). Unfortunately, the hER α G400V sequence also refers to the WT protein sequence in the NCBI database (reference number NP_000116). Conversely, the WT protein sequence in the NCBI database refers to the mutated receptor.

In the course of this study, when we realized that mER α displayed such activity in osteoblasts without additional ligand, we compared the transcriptional output of mER α with that of hER α in transient transfections. We used what we thought was the WT hER α that was obtained by previous lab members from the Chambon laboratory. As a result, hER α did not display any residual activity when no ligand was added (figure 22). We therefore thought that the mER α and hER α had a different ability to convey ligand-independent activity and decided to investigate further the molecular basis of this difference.

Chimeras between hER α and mER α were constructed and the determinant of the ligand-independent activity was found to be entirely localized to a discreet region of domain E (figure 20). The mouse and human receptors differed in four amino acids in

this region when comparing the two WT sequences (figure 21A). However, single and compound mutations of the four divergent amino acids into their mouse or human counterparts failed to affect the activity of the receptor in the absence of ligand (figure 21B). Upon comparing the translation of the cDNA sequences we realized that what we thought to be hER α WT was in fact hER α G400V. G400 is indeed contained in the region where the divergent "ligand-independent activity" was localized. Eventually, the comparison of WT mER α and WT hER α revealed an identical behaviour as far as transcriptional activation is concerned.

It is troublesome that so many laboratories that claim to be using WT hER α refer in their published work to a mutated sequence. This might come from sheer carelessness as most labs might genuinely be working with a WT receptor. It is however also possible that some labs like ours used hER α G400V without being aware of it.

Materials and Methods

Constructs

The ERE-tk-luciferase was a gift from Dr. Paul Webb (Webb *et al.*, 1992). The Renilla expression vector was obtained from Dr. Hentze and contains the *Renilla* luciferase coding sequence cloned between the SmaI and BamHI site of pSG5. hERα (HEG0) and hER α G400V (HE0) were a kind gift of Prof. Chambon (Tora *et al.*, 1989a). The pGL3-PS2 construct has been described before (Metivier *et al.*, 2002b). mERα (pMOR) was a kind gift of Dr MG Parker (Fawell *et al.*, 1990) and was subcloned into the EcoRI and BamHI sites of pSG5 using the following primers:
mERforEcoRI: 5'TCGAATTCATATGACCATGACCCTTCACACC3'
mERrevBamHI: 5' . 5' CCGGATCCTCAGATCGTGTTGGGGAAGCCC 3'
The resulting construct was used as a template to generate all other construct involving mouse ER α.

Truncation mutants:

These mutants were generated by PCR amplification using primers inside the coding sequence of either hER α or mER α. The primers contained adapters that allowed subcloning into the EcoRI and BamHI sites of pSG5. The primers used for each construct were as follows.

mER α deltaA:

mERalphab-ffor: 5' TCGAATTCTCCACCATGCTGGGCGAGGTATACGTG 3'
mERrevbam: 5' CCGGATCCTCAGATCGTGTTGGGGAAGCCC 3'

hER alpha deltaA:

Obtained from Dr. Metivier (Metivier *et al.*, 2000)

mER alpha deltaF:

mERforEcoRI: 5'CGAATTCATATGACCCTTCACACCAAAGCC 3'

mERDeltaFrev: 5’GGATCCTCAGGCATGAAGGCGGTGGGCATCC 3’

hERdeltaF:

hERforEcoRI: 5’ TCGAATTCATATGACCATGACCCTCCACACC 3’

hERDeltaFrev: 5’GGATCCTCACGCATGTAGGCGGTGGGCGTCC3’

mER alpha C-F:

EX2-atg: 5’ TCGAATTCATGATCATGGAGTCTGCCAAG 3’

mERrevbam: 5’ CCGGATCCTCAGATCGTGTTGGGGAAGCCC 3’

hER alpha C-F:

Obtained from Dr. Metivier (Metivier *et al.*, 2000)

<u>HA-tagged mER α</u>

A 5’ or 3’ primer coding for the HA epitope was used to amplify the entire mER α. The resulting product was cloned into the EcoRI and BamHI sites of pSG5. The primers used are:

mERHANterm:

5’CCGAATTCCCACCATGTACCCATACGACGTCCCAGACTACGCTATGACC

ATGACCCTTCACACCAAAGC 3’

mERHACterm:

5’CGCGGATCCTCAAGCGTAGTCTGGGACGTCGTAGGGTAGATCGTGTTGG

GGAAGCCCTCTGC 3’

all the constructs generated were sequence validated.

Site directed mutagenesis

Site directed mutagenesis was performed according to the procedure described in the QuickChange Site-directed Mutagenesis Kit (Stratagene). The primers used to generate the different point mutants are listed below:

mERαN375T

mERN375Tfor: 5’ TTGGGGACTTGACTCTTCATGATCAGGTCC 3’

mERN375Trev: 5’TGGACCTGATCATGAAGAGTCAAGTCCCC 3’

mERαG372V

mERG372Vfor: 5’ AGGCTTTGTCGACTTGAATCTCCATGATCAG 3’

mERG372Vrev: 5’ TCATGGAGATTCAAGTCGACAAAGCCTGGC 3’

mERαG372VN375T

mERG372VN375Tfor: 5’ AGGCTTTGTCGACTTGACTCTCCATGATCAG 3’

mERG372VN375Trev: 5’ TCATGGAGAGTCAAGTCGACAAAGCCTGGC 3’

hERαV368G

hERV368Gfor: 5’ AGGCTTTGGAGATCTGACCCTCCATGATC 3’

hERV368Grev: 5’ TCATGGAGGGTCAGATCTCCAAAGCCTGGC 3’

hERαT371N

hERT371Nfor: 5’ AGGCTTTGTGGATTTAAACCTCCATGATC 3’

hERT371Nrev: 5’ TCATGGAGGTTTAAATCCACAAAGCCTGGC 3’

hERαV368GT371N

hERV368GT371Nfor: 5’ AGGCTTTGGGGATTTAAACCTCCATGATC 3’

hERV368GT371Nrev: 5’ TCATGGAGGTTTAAATCCCCAAAGCCTGGC 3’

mERαR507Q

mERR507Qfor: 5’ AGCAGCATCGCCAGCTGGCTCAGCTCCTTC 3’

mERR507Qrev: 5’ CCGGAATTCTCAGATCGTGTTGGGGAAGCCC 3’

mERαN531S

mERN531Sfor: 5’ ATGGAGCATCTGTACAGCATGAAATGCAAG 3’

mERN531Srev: 5’ TGCATTTCATGCTGTACAGATGCTCCATGCC 3’

hERαR503Q

hERR503Qfor: 5’ TGCAGCAGCAGCACCGCCGGCTGGCCCAGC 3’

hERR503Qrev: 5’ TGGGCCAGCCGGCGGTGCTGCTGCTGCAGG 3’

hERαS527N

hERS527Nfor: 5’ ATGGAGCATCTTTATAACATGAAGTGCAAG 3’

hERS527Nrev: 5’ TTGCACTTCATGTTATAAAGATGCTCCATG 3’

Cell culture

HeLa, MDA-MB231, HepG2, and NIH 3T3 cells were maintained in DMEM supplemented with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and glutamine (2 mM, Invitrogen) at 37ºC in a 5% CO2 incubator. The 2T3 cell line was isolated and cloned from a transgenic mouse containing BMP-2 promoter driving the SV-40 T antigen transgene and has been characterized previously (Ghosh-Choudhury *et al.*, 1996). 2T3 cells were routinely maintained in α-MEM supplemented with 10% FCS supplemented with antibiotics and glutamine never allowed to reach confluence. There differentiation status as assessed by their ALP expression was always determined prior to analysis.

For analysis either by reporter assay or western blot, cells were plated at a density of 1600 cells/cm^2 for 2T3 cells, 5,000 cells/cm^2 for primary calvarial osteoblasts, 8,000 cells/cm^2 for C2C12 cells, For cells that needed to be kept in culture beyond

confluence medium was changed every other day. Two days before analysis, cells were washed twice in PBS and medium was changed to phenol red free medium supplemented with 2.5% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Isolation of primary osteoblasts

Skulls were dissected from 4-6 day-old mice aseptically and placed in 10 ml per skull HBSS with pencillin (100 U/ml; Invitrogen) and streptomycin (100 μg/ml; Invitrogen). The skulls were cleaned from surrounding tissues in a tissue culture hood. Two skulls were placed in 5ml of 0.01% trypsin (Invitrogen), 0.1mM EDTA (4ml of gibco trypsin in 100ml HBSS) and 0.5 mg/ml collagenase P (Roche) and icubated at 37 ºC for 10 min on a rotary shaker. The skulls were thouroughly vortexed and the supernatant discarded. The skulls were incubated for 10 min in 5 ml of the aforementioned trypsin/collagenase mix and this for another 4 cycles. Supernatants from the 4 cycles were pooled into to a 50 ml tube containing 10-15ml of α-MEM supplemented with 10% FCS (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). At the end of the 4 cycles the digested cells were spun down at 400xg and resuspended in 2 ml of FCS. The number of cells was counted using a hemocytometer and diluted to a concentration of 15,000 cells/cm2 and plated in an appropriate culture dish. Once the cells reached confluency they were frozen in FCS/10%DMSO. Primary osteoblast were thawed and amplified. Primary osteoblast were then only passaged once more. The differentiation potential and the purity of the cultures were always assessed by staining the cells for ALP expression.

Assessment of cell number

Cell number was determined in triplicate wells at various time points. The cell layer was washed with PBS, and the cells were then incubated with 0.1-0.5 ml of 0.05% trypsin-25 mM EDTA (Gibco) at 37ºC for 10-40 min (until the cells in the wells visibly rounded up). An equal volume of FCS was then added and the cells were then dispersed to a single cell suspension with 25 up/down strokes using a 1 ml automatic pipetting device. Cell number was determined using a hemocytometer.

Protein isolation and western blot analysis

Cells were washed once in PBS and scraped into 1 ml of ice-cold PBS. Cells were peleted at 400xg for 4 min at 4ºC. 2T3 cells and primary osteoblasts were grown in duplicates and cell number was assessed in the duplicate culture as mentioned above. The supernatant was carefully removed and the cells pellet was resuspended in 30 ul/600,000cells of RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na-Deoxycholate, 0.1% SDS) with the protease inhibitor cocktail Complete Mini (Roche). Cells were allowed to lyse at 4ºC for 30 min and spun at 14,000 rpm for 15 min on a tabletop centrifuge at 4ºC. The supernatant was used immediately or frozen at -70ºC for later use. Proteins amount were quantified by Bradford assay (BioRad). Isolated uteri from 6 week-old female mice frozen in liquid N_2 and kept at -70ºC before use. Protein from uteri wAS then powdered in liquid nitrogen using pestle and mortar. The powdered tissue was transferred into RIPA buffer and the samples were processed as described for cultured cells.

Aliquots of 100 μg protein were resolved on a 10% SDS-PAGE gel alongside the rainbow protein marker (Invitrogen). To separate the doublet of ER α, the gels were run for 24h before blotting, for standard western blot analyses, gel were run over

shorter time periods. Proteins were transferred on PVDF membranes (Millipore) overnight at 50V.

Membranes were blocked in 5% milk PBS-T (PBS and 0.05% Tween 20, Sigma) for 20 min. Following blocking membranes were incubated with primary antibodies in 5% milk PBS-T. The primary antibodies used in this study and the concentrations used were as follows: MC-20 (1:2000, Santa Cruz), H-184 (1:2000, Santa Cruz), mouse monoclonal anti β-Actin (1:5000, Sigma), H-11 (1:2000, Santacruz). After 1-4h incubation the primary antibodies were removed and the membrane washed three times with PBS-T. Secondary antibodies in 5% milk PBS-T were added to the membrane for 1.5h. The horse-radish peroxidase conjugates were purchased from DiaNova.

Transfections

Subconfluent and confluent cells were transfected with Fugene 6 reagent according to the manufacturer's instructions (Roche). If cells were to be used for western analysis, they were grown in 6-well plates (Nunc). Cells used for luciferase assays were grown in 24-well plates (Nunc). Transfections were performed as follows. 20 min before transfections cells were washed twice in PBS and growth medium was changed to phenol red free medium with 2.5% FCS and antibiotics. Cells were placed back in the incubator and the transfection mix prepared. 0.3 ug/well was transfected in 24-well plates while 1.5 μg/well was transfected in 6-well plates. The ratio μg DNA/ μl Fugene 6 was kept at 1:6 and the ratio Fugene 6/ serum free medium at 1:35. For luciferase assays the ratio of the different plasmids were: Estrogen receptor construct or empty vector: ERE-tk-luciferase: renilla, 1:50:0.06. For westerns, ERE-luciferase and renilla were replaced by empty vector.

On the following day medium was replaced for fresh phenol red free medium with 2.5% FCS containing the appropriate concentrations of erthanol vehicle estradiol (Sigma), ICI 182,780 (?). MG132 (Sigma) was added concomitantly.

Luciferase assay

Cultured cells were washed twice in PBS. Apart from subconfluent 2T3 cells which were lysed in 80 µl active lysis buffer (Roche), other cells were lysed in 100 µl of lysis buffer. Cells were allowed to lyse at room temperature for 3h in the case of 2T3 cells and for 1h for every other cell line. 10 µl of each lysate was used in the Dual Luciferase assay (Promega) with 10s emission measurements and 2s delays after the injection of 50 µl luciferase assay reagent and injection of 50 µl Stop&Glow reagent. Measurement was performed in 96-well micrplates (nunc) using the EG&G Berthold Microplate Luminometer LB 96V.

RNA and DNA isolation

Uteri from 6-week old female mice were frozen in liquid N2 and kept at –70ºC until use. Uteri were ground with pestle and mortar in liquid N2 and the powdered tissue was transferred into TRIzol (Invitrogen). The RNA and DNA isolation procedure were performed as recommended by the manufacturer. DNA was resuspended in water and concentration was determined by the absorbance at 260 nm. RNA was resuspended in water and kept at -70ºC until it was used. Concentration was determined by absorbance at 260 nm and quality of the RNA was assessed by agarose gel electrophoresis.

B-galactosidase reporter gene assay

Primary osteoblasts from the ERE-tk-bet-Galactosidase ERIN mice (Nagel *et al.*, 2001) were isolated as described above. The detection of β-galactosidase was performed with the Galacto-star™ chemiluminescent reporter gene assay (Tropix). Cells were plated in 6-well plates. Subconfluent cells were used the day after for the assay while cells were kept up to 5 days after confluency was reached for differentiated cells. Cells were rinsed once with PBS and subsequently scraped into 100 µl of lysis buffer for subconfluent cells or 500 µl for confluent cells and cells were incubated at room temperature for 10 min. 10 ul of each lysates were transferred into 96-well microplates (nunc) and measurement was performed after addition of 100 µl of substrate with a EG&G Berthold Microplate Luminometer LB 96V for 10 sec.

Osteogenic differentiation of 2T3 and primary calvarial osteoblasts

2T3 cells and primary calvarial osteoblasts were plated at 1600 cells/cm2 and 5,000 cells/cm2 respectively in α-MEM supplemented with 10% FCS supplemented with antibiotics and glutamine. For cells with an undifferentiated phenotype, cells were washed twice with PBS and the medium was changed the day after to phenol red free medium with 2.5% charcoal-stripped FCS, antibiotics and glutamine. Two days afterwards the cells were still subconfluent and undifferentiated as assessed by their ALP negative phenotype. For differentiation, cells were kept in 10% α-MEM until 2 days prior to analysis when the medium was switched to phenol red free medium with 2.5% charcoal-stripped FCS. Differentiation was assessed by ALP staining.

TGF β

Differentiation of C2C12 cells

C2C12 cells were plated at 8,000 cells/cm2 in DMEM with 10% FCS, antibiotics and glutamine. Cells reached confluence two days afterwards. The medium was then changed to DMEM with 5% FCS. Cells spontaneously differentiated into multinucleated myoblasts as soon as three days after confluency was reached under these conditions. To induce osteogenic differentiation, BMP2 (300 ng/ml, Genetics institute) was added on the day confluency was reached and thereafter every other day. Osteogenic differentiation was assessed by ALP staining.

Alkaline phosphatase assay

Cells were plated at the appropriate density in 24-well plates (Nunc). The Alkaline Phosphatase detection kit was used and performed according to the manufacturer's instructions (Sigma-diagnostics). Cells were washed twice in PBS directly in the 24-well plates and fixed in 1ml of fixing solution. Cells were then washed twice in water and incubated with 1ml of substrate solution for 15 min at room temperature in the dark. Following staining, the cells were washed twice with water and air-dried.

Mineralized Bone Matrix Formation Assay

Bone cell differentiation was monitored using a mineralized matrix formation assay as described previously (Chen *et al.*, 1998).Von Kossa stain of mineralized bone matrix was performed as follows. The cell cultures were washed with PBS twice, fixed in phosphate-buffered formalin for 10 min and then washed with water, and serially dehydrated in 70, 95, and 100% ethanol, twice each, and then air dried. The plates were rehydrated from 100 to 95 to 80% ethanol/water before staining. The water was removed, a 2% silver nitrate (Sigma) solution was added, and then the plates were

exposed to sunlight for 20 min after which the plates were rinsed with water. 5% sodium thiosulfate (Sigma) was added for 3 min and the plates were then rinsed with water. The modified van Gieson stain was then used as a counterstain after the von Kossa stain. The unmineralized collagen matrix can be recognized by the yellow-red van Gieson stain. The acid fuchsin solution (5 parts of 1% acid fuchsin (Sigma), 95 parts of picric acid (Sigma), and 0.25 part of 12 M HCl) was added for 5 min. The plates were washed with water and then with 2× 95% ethanol, 2× 100% ethanol and then air dried.

Quantitative RT-PCR

RNA were prepared form sampled cells by the TriZol Reagent (Invitrogen). Reverse-transcription using poly-dT oligos (Roche) was then performed on 8 μg RNA treated with DNAse (Roche). The 5’ to 3’ sequences of the primers used in the PCR (MWG GmbH) were:

GAPDH:

GAPDHup 5' GAGGCCGCCGCACGACAACCGCA 3'

GAPDHdown 5' ACGGTGGGGAAGACTGTCCTGCCTG 3'

mER α:

ex5-1u: 5’ AGTGTGCCTGGCGGAGATTCTGATGATTG

ex6-1: 5’ AGATCTCCACCATGCCTTCCACAC 3’

Quantitative PCR were performed using SybrGreen (Molecular Probes) as marker for DNA amplification on a SmartCycler (Eurogentec) with 40 cycles. Detectable amplification of specific products arose between 13 to 28 cycles.

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