Stem Cells and Cancer Stem Cells 6
Therapeutic Applications in Disease and Injury

M.A. Hayat
Editor

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Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena.

Richard J. Reed, MD

One Point of View

All small tumors do not always keep growing, especially small breast tumors, testicular tumors, and prostate tumors. Some small tumors may even disappear without a treatment. Indeed, because prostate tumor grows slowly, it is not unusual that a patient may die at an advanced age of some other causes, but prostate tumor is discovered in an autopsy study. In some cases of prostate tumors, the patient should be offered the option of active surveillance followed by PSA test or biopsies. Similarly, every small kidney tumor may not change or may even regress. Another example of cancer or precancer reversal is cervical cancer. Precancerous cervical cells found with Pap test, may revert to normal cells. Tumor shrinkage, regression, reversal, or stabilization is not impossible.

Another known example of cancer regression is found in pediatric neuroblastoma patients. Neuroblastoma shows one of the highest rates of spontaneous regression among malignant tumors. In addition to the well-known spontaneous regression in stage 4S disease, the high incidence of neuroblastoma remnants found during autopsy of newborns suggest that localized lesions may undergo a similar regression (Guin et al. 1969). Later studies also indicate that spontaneous regression is regularly seen in infants with localized neuroblastoma and is not limited to the first year of life (Hero et al. 2008). These and other studies justify the "wait and see" strategy, avoiding chemotherapy and radiotherapy in infants with localized neuroblastoma, unless MYCN gene is amplified. Infants with nonamplified MYCN and hyperdiploidy can be effectively treated with less intensive therapy. Infants with disseminated disease without MYCN have excellent survival with minimal or no treatment. Another example of spontaneous shrinkage and loss of tumors without any treatment is an intradural lipoma (Endoh et al. 1998).

Overtreatment

An example of unnecessary surgery is the removal of all the armpit lymph nodes after a biopsy when a sentinel node shows early stage breast cancer; removal of only the sentinel node may be needed. Limiting the surgery to the sentinel node avoids painful surgery of the armpit lymph nodes, which can have complications such as swelling and infection (such limited surgery is

already being practiced at the Memorial Sloan-Kettering Cancer Research Center). Radiation-induced second cerebral tumors constitute a significant risk for persons undergoing radiotherapy for the management of cerebral neoplasms. High-grade gliomas are the most common radiation-induced tumors in children (Pettorini et al. 2008). The actual incidence of this complication is not known, although it is thought to be generally low.

Prostate cancer treatment is an example of overtreatment. Serum prostate specific antigen (PSA) testing for the early detection of prostate cancer is in wide use. However, the benefit of this testing has become controversial. The normal cut-off for serum levels of PSA is 4 ng/ml, so any man presenting a PSA above this level is likely to require rectal biopsy, but only in 25% of men with serum levels of PSA between 4 ng and 10 ng/ml have cancer (Masters 2007). The PSA threshold being used for biopsy ranges between 2.5 and 3.4 ng/ml. Up to 50% of men presenting with prostate cancer have PSA levels within the normal range. It is apparent that screening of prostate cancer using PSA has a low specificity, resulting in many unnecessary biopsies, particularly for gray zone values (4 ng–10 ng/ml). According to one point of view, the risks of prostate cancer overdetection are substantial. In this context, overdetection means treating a cancer that otherwise would not progress to clinically significant disease during the lifetime of the individual. Overdetection results in overtreatment. The risk of death for men in the United States between the ages of 55 and 74 years due to cardiovascular disease surpasses that of prostate cancer. Cardiovascular disease is the most common of the chronic non-communicable diseases that impact global mortality. Approximately, 30% of all deaths worldwide and 10% of all healthy life lost to disease are accounted for by cardiovascular disease alone. The advantages and limitations of PSA in diagnosing prostate cancer were reviewed by Hayat (2005, 2008).

A significant decrease in the risk of prostate cancer-specific mortality is observed in men with few or no comorbidities. Indeed, active surveillance in lieu of immediate treatment (surgery or radiation, or both) is gaining acceptance. Most men with prostate cancer, even those with high-risk disease, ultimately die as a result of other causes (Lu-Yao et al. 2009). Debate on this controversy is welcome, but narrow opinions and facile guidelines should lead to fact and new information; men worldwide deserve it (Carroll et al. 2011). Automatic linking positive diagnosis with treatment, unfortunately, is a common clinical practice. Unfortunately, even men who are excellent candidates for active surveillance in the United States often undergo some treatment. Deferment of treatment is advised in men with low-risk disease.

In addition to unwanted side effects of some drugs, excipients (e.g., propylene glycol, menthol) may pose safety concerns in some patients. Excipients are defined as the constituents of the pharmaceutical formulation used to guarantee stability, and physicochemical, organoleptic and biopharmaceutical properties. Excipients frequently make up the majority of the volume of oral and parenteral drugs. Not all excipients are inert from the biological point of view. Although adverse drug reactions caused by the excipients are a minority of all adverse effects of medicinal products, the lack of awareness of the possible risk from excipients should be a concern for regulatory agencies, physicians, and patients (Ursino et al. 2011). Knowledge of the potential side effects of excipients is important in clinical practice.

The pertinent question is: Is it always necessary to practice tumor surgery, radiotherapy, or chemotherapy? Although the conventional belief is that cancer represents an "arrow that advances unidirectionally", it is becoming clear that for cancer to progress, it requires cooperative microenvironment (niche), including immune system and hormone levels. However, it is emphasized that advanced (malignant) cancers do not show regression, and require therapy.

First whole genome sequences of prostate tumors were recently published online in Nature journal (vol. 470: 214–220, 2011). This study revealed that rather than single spelling errors, the tumor has long "paragraphs" of DNA that seem to have broken off and moved to another part of the genome (rearrangement of genes), where they are most active. These portions of DNA contain genes that help drive cancer progression. The mutated genes involved include PTEN, CADM2, MAG12, SPOP, and SPTA1. This information may lead to the development of more efficient, less invasive ways to diagnose and treat this cancer. Such information, in addition, should lead to personalized therapeutics according to sequencing results of different gene mutations or chromosomal rearrangements. The urgent need of such studies becomes apparent considering that more than 200,000 new prostate cancer cases and 32,000 deaths are reported annually in the United States. In the light of the inadequacy of standard treatments of malignancy, clinical applications of the stem cell technology need to be expedited.

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Preface

Stem Cells is nature's indispensable gift to multicellular organisms.

This is volume 6 of the seven-volume series, STEM CELLS AND CANCER STEM CELLS: Therapeutic Applications in Disease and Tissue Injury. A stem cell is defined as a cell that can self-renew and differentiate into one or more specialized cell types. A stem cell may be pluripotent, which is able to give rise to the endodermal, ectodermal, and mesodermal lineages; an example is embryonic stem cells. A stem cell may be multipotent, which is able to give rise to all cells in a particular lineage; examples are hematopoietic stem cells and neural stem cells. A stem cell may be unipotent, which is able to give rise to only one cell type; an example is keratinocytes.

A cancer stem cell is a cell type within a tumor that possesses the capacity of self-renewal and can give rise to the heterogeneous lineages of cancer cells that comprise the tumor. In other words, a cancer stem cell is a tumor initiating cell. A unique feature of a cancer stem cell is that although conventional chemotherapy will kill most cells in a tumor; cancer stem cells remain intact, resulting in the development of resistance of therapy. All of these types of stem cells are discussed in this volume. Vast therapeutic applications of the following specific stem cells in disease and tissue injury are discussed: human embryonic stem cells, human mesenchymal stem cells, germ cell-derived pluripotent stem cells, induced pluripotent stem cells, human umbilical cord blood-derived stem cells, breast tumor stem cells, and hematopoietic stem cells.

As stated above, given that human embryonic stem cells possess the potential to produce unlimited quantities of any human cell type; considerable focus has been placed on their therapeutic potential. Because of the pluripotency of embryonic stem cells, they have been used in various applications such as tissue engineering, regenerative medicine, pharmacological and toxicological studies, and fundamental studies of cell differentiation. The formation of embryoid bodies, which are three-dimensional aggregates of embryonic stem cells, is the initial step in the differentiation of these cells. Such embryoid body culture has been widely used as a trigger for the in vitro differentiation of embryonic stem cells.

The basic capacity of self-renewal of human embryogenic stem cells is explained. The role of TGF-β in the propagation of human embryonic stem cells is discussed, so is the differentiation of these cells into neurons, hepatocytes, cardiomyocytes, and retinal cells. Molecular signaling pathways that modulate mesenchymal stem self-renewal are discussed. The regenerative potential of stem cells and their mesenchymal progeny is explained. Clinical

applications of mesenchymal stem cells are reviewed, and their use for treating cancer patients, diabetes, and neurodegenerative pathologies is detailed.

Donor policies for hematopoietic stem cell transplantation are explained. The usefulness of allogenic stem cell transplantation of patients with chronic lymphocytic leukemia is underscored. Clinical use of hematopoietic stem cells for patients with myeloma is explained. Response of hematopoietic stem/progenitor cells to chemotherapy is clarified. Therapeutic applications of stem cells and induced pluripotent stem cells in treating Parkinson's disease are presented.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against terrible human disease and injury. It is difficult for a single author to discuss effectively the complexity of diagnosis, therapy, including tissue regeneration. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of cancer cure and tissue regeneration. I hope these goals will be fulfilled in this and other volumes of the series. This volume was written by 108 contributors representing 17 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the readers in these important areas of disease and injury. I respect and appreciate the hard work and exceptional insight into the nature of cancer and other disease provided by these contributors. The contents of the volume are divided into five subheadings: Embryonic Stem Cells, Mesenchymal Stem Cells, Hematopoietic Stem Cells, Other Types of Stem Cells, and Parkinson's Disease for the convenience of the reader.

It is my hope that subsequent volumes of the series will join this volume in assisting in the more complete understanding of the causes, diagnosis, and cell-based treatment of major human diseases and debilitating tissue/organ injuries. There exists a tremendous, urgent demand by the public and the scientific community to address to cancer diagnosis, treatment, cure, and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating deadly malignancies over military superiority.

I am thankful to Dr. Dawood Farahi and Dr. Kristie Reilly for recognizing the importance of medical research and publishing through an institution of higher education.

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Part I

Embryonic Stem Cells

Propagation of Human Embryonic Stem Cells: Role of TGF β

1

Prasad Pethe and Deepa Bhartiya

Abstract

Human embryonic stem cells (hES) have tremendous application in several areas of research including regenerative medicine, developmental biology, drug screening, and drug discovery. Transforming Growth Factor (TGF) β and its family of proteins have an indispensible role in genesis and maintenance of various organs and cancer and are also used for the propagation of embryonic stem cells. In this review, we discuss the TGF β family members, and their signalling, and delve into the molecular mechanism by which TGF β proteins may regulate the propagation of human embryonic stem cells.

Introduction

Embryonic stem cells (ES) are pluripotent and self-renewing cells derived from inner cell mass (ICM) of blastocysts. Human ES cells were initially derived on inactivated mouse embryonic fibroblast (MEF) in a serum containing medium (Thomson et al. 1998). Subsequently many research groups including ours, successfully derived hES cell lines on feeder cells of human origin (Kumar et al. 2009) to avoid the risk of transfer of potential animal pathogens. hES cells growing on feeder cell layers have the ability to self-renew, propagate indefinitely and differentiate into all three germ layers viz, ectoderm, mesoderm, and endoderm. This ability of hES cells makes them a candidate for cell-based therapies for several disorders such as alzheimer's disease, diabetes, cardiovascular diseases, and spinal cord injuries, for which available therapies have had limited success. The prerequisite for their use for cell therapy is propagation of hES cells in large numbers using a defined culture medium followed by their differentiation into desired cell type. However, propagation of hES cells in a defined culture medium remains a challenge and several research groups have investigated the role of feeder cells and the cytokines secreted by them in order to understand hES cell propagation.

Proteomics and microarray studies aimed at identifying the key signaling molecules involved in propagation of hES cells, identified TGF β family proteins as one of the key candidates, along with fibroblast growth factor (FGF), Wnt proteins, and extracellular matrix components

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(ECM) (Kumar et al. 2010; Prowse et al. 2005). Supplementation of culture media with TGF β proteins during serum-free culturing of hES cells maintained them in undifferentiated state (Eiselleova et al. 2008; Watabe and Miyazono 2009) and inhibition of TGFβ/nodal/activin pathway leads to differentiation of hES cells (James et al. 2005). Thus it is inferred that TGFβ signalling pathway is essential for hES cell self renewal.

Embryonic stem cells share several characteristics with cancer cells; among them is requirement of TGF β for growth and differentiation (Mishra et al. 2005). It is interesting to draw an analogy between cancer cells and surrounding stromal fibroblasts with embryonic stem cells and surrounding feeder fibroblasts. TGF β has been implicated to have a dual role during carcinogenesis (Roberts and Wakefield 2003) and acts to promote tumor progression as well as tumor suppression. It induces epithelial-mesenchymal transition, and stromal cells at the tumor front differentiate into myofibroblasts and modify the tumor microenvironment. This stromal-epithelial cross-talk results in further growth at the tumor front; thus, promoting tumor growth and progression. Similarly, available reports suggests that TGF β plays dual role during both differentiation and proliferation of hES cells (Watabe and Miyazono 2009; Puceat 2007; Valdimarsdottir and Mummery 2005), and feeder fibroblasts that support proliferation of the cells have a tendency to differentiate into myofibroblasts (Kumar et al. 2010). Indeed ES cells are considered as a good *in vitro* model to understand role of TGF β signalling during proliferation and differentiation. The present review focuses on the role played by TGF β in propagation of hES cells and we have purposely referenced review articles when possible to amplify the reference base.

TGF Beta Superfamily

TGF β superfamily of proteins discovered in the 1980s, contains growth factors that elicit varied effects, depending on the cell type, and amount and duration of exposure (Valdimarsdottir and Mummery 2005). TGF β is a pleiotropic growth factor with several functions including embryonic development, cell growth, differentiation, migration, extracellular matrix deposition, apoptosis, homeostasis, tissue injury, inflammation, cancer, and stem cell biology. Crucial role played by TGF β in the regulation and modulation of various pathological diseases has been recently reviewed (Aihara et al. 2011).

TGF β superfamily consisting of more than 40 members, are synthesized as large precursor molecules which are cleaved at the RXXR site during translation to release a 110–140 amino acid C-terminal segment. Each monomer is composed of β strands containing seven cysteine residues. The strands are interconnected by three disulphide bonds formed between six cysteine residues, to form a rigid structure called "cysteine knot" (Shi and Massague 2003), whereas, the seventh cysteine is involved in the formation of a disulphide bond with another monomer, forming a homodimer or heterodimer of TGF β family molecule, and thus making up the active TGFβ ligand (Kingsley 1994).

Based on sequence similarity and signalling pathways they activate, the TGF β family members can be classified into two subfamilies (i) TGFβ (Transforming Growth Factor)/Activin/Nodal and (ii) BMP (Bone Morphogenic Protein)/GDF (Growth Differentiation Factor)/MIS (Muellerian Inhibitory substance). There are several isoforms of TGFβ superfamily members. BMP subfamily has more number of members than TGFβ/Activin/Nodal (Kingsley 1994).

Signalling Cascade

For TGF β ligands to induce change in a target cell, they must interact with corresponding receptor to transmit the signal to the nucleus; thus, leading to transcriptional modulation of the target genes. TGF β receptors have N- terminal ligand binding domain, a transmembrane region and a C-terminal domain with serine/threonine kinase activity. Broadly, the TGF β receptors present on hES cell surface can be divided into two classes namely Type I receptors and Type II receptors (Wu and Hill 2009; Shi and Massague

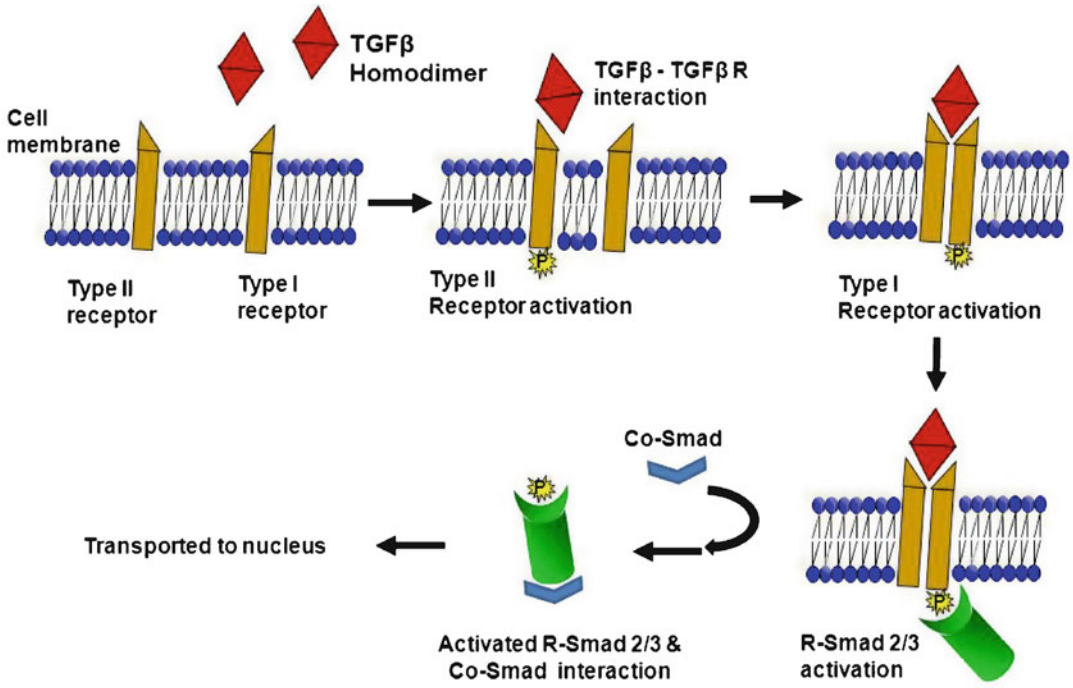

Fig. 1.1 TGFβ/Activin/Nodal signalling by activation of Smad 2 and 3 effector proteins in human embryonic stem cells

2003; Massague 1998). There are seven Type I receptors and five type II receptors, different combinations of ligand receptor interactions can occur; thus, creating diversity in the signal transduction (Derynck and Zhang 2003). TGF β/Activin subfamily shows higher affinity for type II receptors compared to type I receptors, while BMP subfamily shows greater affinity towards type I receptor (Massague 1998).

TGF β ligands binds first to type II followed by interaction with type I receptors this binding initiates a cascade of reactions beginning with phosphorylation of the GS domain of the type I receptors by the type II receptor kinase causing activation of type I receptor. The serine/threonine kinase of activated type I receptors then phosphorylates proteins of various signalling pathways, thereby bringing about varied effects (Figs. 1.1 and 1.2). The receptor ligand complex activates proteins such as ShcA, Grb, SoS and JNK proteins of the p38 MAPK signalling (Lee et al. 2007; Shi and Massague 2003). TGF β signaling also activates PAR6 by phosphorylation which initiates epithelial mesenchymal transition (Ozdamar et al. 2005). This ability is of significance in the case of cancer cells that become metastatic.

The Smad proteins are the effector molecules of the TGF β signalling. They lie downstream of the TGF β receptors and are the most studied in the context of embryonic stem cells. They were first indentified as mediators of TGF β signalling in *Drosophila*, later its orthologs were discovered in vertebrates (Shi and Massague 2003). There are three classes of Smad proteins in hES cells, viz. (Receptor regulated Smad) R-Smads 1,2,3,5 and 8, (Common Mediator) Co-Smad 4 and (Inhibitory Smad) I-Smad 6 and 7 (Valdimarsdottir and Mummery 2005). Smad proteins comprise of two conserved domains, the N-terminal MH1 domain and the C-terminal MH2 domain. Both domains have a proline linker sequence which has a MAP kinase phosphorylation site, and phosphorylation of this region can negatively

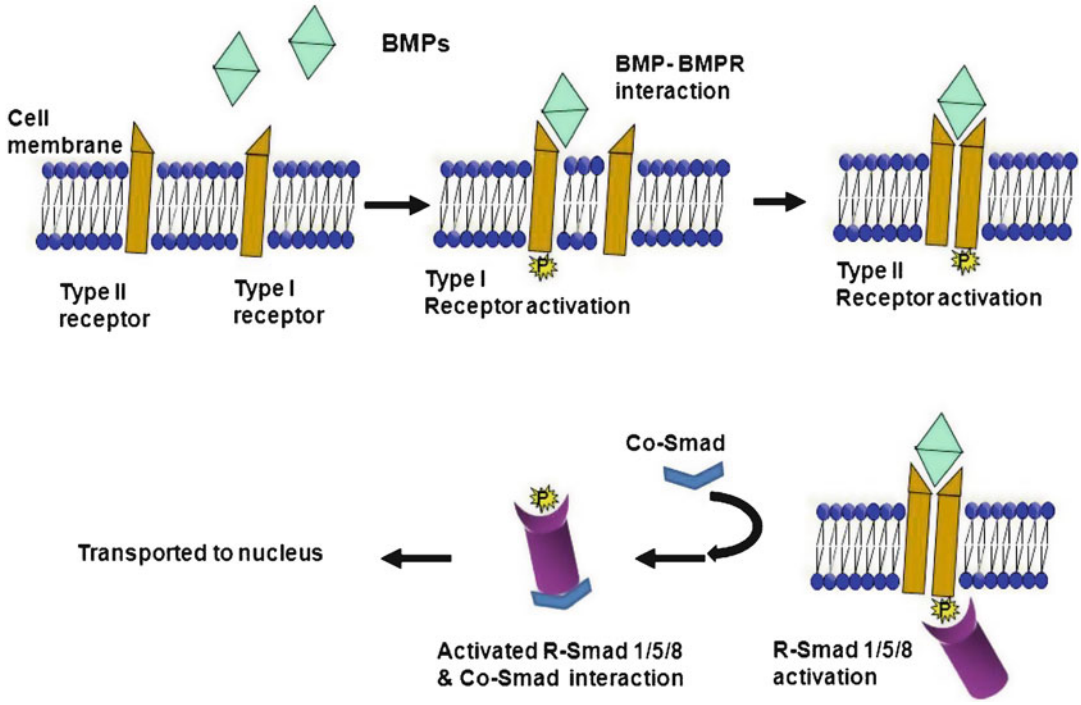

Fig. 1.2 BMP–BMPR interaction and activation of Smad 1/5/8 proteins leading to differentiation

regulate Smad activity (Zhang and Derynck 1999). The MH2 domain is highly conserved in all Smad proteins, and is responsible for receptor recognition, formation of homo-heterodimeric Smad complexes, and nuclear transport. While MH1 domain helps in the interaction with nucleoprotein complex and sequence specific DNA binding (Valdimarsdottir and Mummery 2005; Shi and Massague 2003). The R-Smads contain a characteristic SXS region in the MH2 domain, this region is phosphorylated by type I receptor kinase leading to its activation, but this region is absent in Co-Smad 4 (Shi and Massague 2003).

Embryonic Stem Cell Propagation and Self Renewal

James et al. (2005) demonstrated that the hES cells cultured using MEF conditioned media has phosphorylated Smad 2/3 proteins in the nucleus implying that TGF β/activin/nodal pathways were activated. Using a synthetic inhibitor SB431542, TGF β signalling and subsequent Smad 2/3 activation was inhibited. The inhibitor reduced the Oct4 expression in hES cells grown in MEF conditioned media. The results from this study prompted use of Activin A for feeder free culturing of hES cells. Low concentrations of Activin A could maintain the levels of pluripotency transcription factors Oct4 and Nanog. On the other hand addition of BMP 4 to the growth media alone or after inhibition of TGF β/activin/nodal signalling lead to Smad 1/5 phosphorylation and differentiation (Xiao et al. 2006). These results are intriguing and demonstrate that TGF beta family of proteins exerts dual action during hES cells proliferation and differentiation possibly through different signaling mechanisms, similar to its well documented role in tumor biology. For undifferentiated propagation of hES cells the TGF β/activin/nodal signalling is essential while BMP signalling initiates differentiation. Phosphorylated Smad 2/3 are effectors of TGF β/activin/nodal signalling while phosphorylated Smad 1/5/8 mediates BMP signalling.

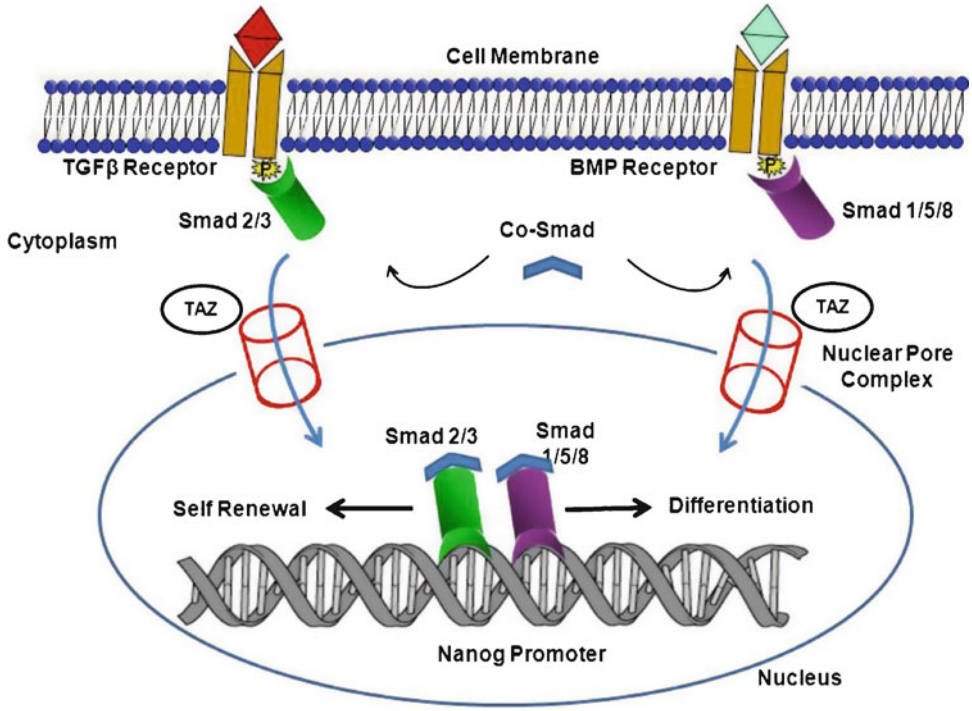

Fig. 1.3 Mechanism of human embryonic stem cell self-renewal regulated by the TGFβ family proteins

Subtle differences in the growth factor requirement for *in vitro* expansion of mES and hES cells exist. Mouse ES cells can be derived and maintained in the presence of LIF and BMP, which act via Stat3 and Smad 1 pathway. In contrast LIF is not crucial for hES cells and BMPs promote differentiation into trophoectoderm. This could possibly be, because the hES cells possibly have a germ cell origin (Zwaka and Thomson 2005) and resemble more closely to mouse epiblast derived ES cells rather than mES cells derived from inner cell mass of blastocyst. This also explains the greater similarity in the requirement of extrinsic factors for *in vitro* maintenance of hES cells and mouse epiblast stage derived pluripotent stem cells (Jiang and Ng 2008).

One of the main growth factors added to the culture medium for proliferation of hES cells is basic fibroblast growth factor (bFGF) is upstream of the TGF β signalling pathway and when added exogenously to the MEF conditioned media regulates the expression of TGF β family members in the feeder cells (Greber et al. 2007; Eiselleova et al. 2008). Thus for expansion and propagation of hES cells, nature of feeder cells used for preparing conditioned media and the substrate for culturing dictates the mechanism of hES self renewal. Our group (Kumar et al. 2010) has studied the gene expression of supportive (13.5 dpc) and non-supportive (18.5) mouse feeder cells as well as human feeder cells using microarray and scanning electron microscopy. We demonstrated that besides secreting cytokines like TGF β, the human feeder cells assume myofibroblast morphology that helps in propagation of hES cells in undifferentiated state.

TGF β signalling pathway has been shown to be essential for self renewal of hES cells. Many aspects of how this pathway helps in propagation of hES cells were not fully understood. The TGF β/Activin/Nodal signalling led to activation of Smad 2/3 by phosphorylation but caused inhibition of Smad 1/5/8. Xu et al. (2008) carried out experiments that help piece together TGF β/Activin/Nodal signalling and maintenance of pluripotency in hES cells. Chromatin Immunoprecipitation (ChIP) experiments were