Mini review

Transforming Growth Factor type β and Smad family signaling in stem cell function

Eve Seuntjens a,b,1, Lieve Umans a,b,2, An Zwijsen c,b,2, Maurilio Sampaolesi d,3, Catherine M. Verfaillie e,4, Danny Huylebroeck a,b,*

a Laboratory of Molecular Biology (Celgen) of the Center for Human Genetics, University of Leuven, Flanders Institute of Biotechnology (VIB), Campus Gasthuisberg, Building Ondr&Nav1 Box 812, Herestraat 49, B-3000 Leuven, Belgium
b Department of Molecular and Developmental Genetics (VIB11), Flanders Institute of Biotechnology (VIB), Campus Gasthuisberg, Building Ondr&Nav1 Box 812, Herestraat 49, B-3000 Leuven, Belgium
c Laboratory of Developmental Signalling of the Center for Human Genetics, University of Leuven, Flanders Institute of Biotechnology (VIB), Campus Gasthuisberg, Building Ondr&Nav1 Box 812, Herestraat 49, B-3000 Leuven, Belgium
d Laboratory of Translational Cardiomyology, Interdepartmental Stem Cell Institute (SCIL), University of Leuven, Campus Gasthuisberg, Building Ondr&Nav1 Box 804, Herestraat 49, B-3000 Leuven, Belgium
e Interdepartmental Stem Cell Institute (SCIL), University of Leuven, Campus Gasthuisberg, Building Ondr&Nav1 Box 804, Herestraat 49, B-3000 Leuven, Belgium

1. Targeting and exploiting pleiotropic TGFβ signaling

1.1. TGFβ family signaling as a major regulator of embryogenesis

Over the last 20 years developmental biologists have revolutionized their field through the identification and functional analysis of the genetic and molecular mechanisms that underlie the sudden or progressive change in the fate of cells in the embryo. This change takes place during the commitment from pluripotent stem cells to germ-layer committed and allocated cells, and subsequent generation of tissue-specific stem cells as well as their final differentiated progeny. Ultimately, this yields perfectly shaped and functional organs.

A number of families of polypeptide growth factors influence cell responses via their receptor-activated intracellular signaling pathways that converge on specific target genes, including genes encoding key transcription factors (TFs), whose
expression is then modified. Therefore, these growth factors steer cell fate and determination primarily through a combination of their paracrine and thus extrinsic control with intrinsic regulation of the expression and activity of key TFs in the target cell. Soluble ligands of the Transforming Growth Factor type β (TGFβ) family, encoded by 33 genes in human, including TGFβs itself, Nodal and Activin proteins, and Bone Morphogenetic Proteins (BMPs) orchestrate diverse processes during embryogenesis. They act together with an increasing number of identified ligand-binding/modulating proteins (mainly for BMPs) and specific proteases for these, few signaling receptors and co-receptors, and few intracellular effector Smad proteins (i.e. the receptor-regulated TGFβ/Nodal/Activin Smad2 and Smad3, and the BMP-Smads Smad1, Smad5 and Smad8). Remarkably, the Smad canonical signaling pathway involves very high numbers of Smad-interacting proteins (SIPs), many of which are TFs or scaffolding proteins. This signaling system establishes the basic body plan of the embryo, patterns many of its structures and tissues, and drives morphogenetic processes in the embryo and fetus, including its growth. It also operates during post-natal development and, by extension, in homeostasis and tissue repair [1,2].

At the cellular level the TGFβ system is involved in the regulation of cell proliferation and growth arrest, pluri potency and differentiation, cell survival and apoptosis, and cell migration and cell–cell (de)adhesion. These activities depend on cellular context, i.e. the stage of the target cell, the local environment, and the identity and dosage of the ligand [3–6]. Further regulatory mechanisms include the composition and assembly mode of the receptor complexes and the spatial–temporal control of the signaling by the latter, and the degradation through endocytosis of liganded receptors [7–10] as well as degradation of the ligand itself [11]. Yet other controls occur through ubiquitination of effector proteins [12,13], changes in the dynamics of the nucleocytoplasmic shuttling of Smads [14], and the posttranslational modification of Smads (e.g. acetylation; phosphorylation, including of the linker domain, ref. [15]; [16]).

1.2. TGFβ as cause of disease and as therapeutic target

Inappropriate production of TGFβ factors or aberrant interpretation of TGFβ signals leads to chronic and congenital diseases, including fibrosis, cancer, and disorders of cartilage, bone, muscle, fat, vessel wall and the immune system, revealing crucial functions of the TGFβ system in normal tissue homeostasis (for recent reviews, see Refs. [17–23]). Through the use of a combination of biochemistry, structural biology, genetics, cell biology, and experimentation in animal models and with tissues or cells derived from these, it has become clear that TGFβ family signaling is not only of fundamental but also medical relevance for most – if not all – body systems. Hence, it represents a challenging and likely unavoidable target for future therapeutic intervention [24].

This all-embracing signaling system is complex and subject to tight regulation at multiple levels of the canonical Smad pathway [25], making pharmaceutical and biotech feel reluctant to target it. However, they know that – for example – the BMP pathway is relevant to cartilage/bone metabolism, but also to a growing list of soft tissues and organs, where their malfunction or pathology is observed in patients and/or illustrated in conditional knockout mouse models. These include kidney (injury, ischemia; [26,27]), heart (induction, morphogenesis, conduction, valves; [28,29]), endothelial cells (angiogenesis; [30]), ovarian cancer (granulosa cell tumors; [31]), (in)fertility in females (interaction between the oocyte and follicle cells) and males (persistent Müllerian duct) [32–34], metastasis in breast cancer [35], brain tumors (gliomas; [36,37]), cranio–facial development [38,39], remyelination and repair of injured spinal cord [40,41], and adult neurogenesis in the brain (lateral ventricle; [42]). TGFβ itself regulates wound healing and promotes scarring and fibrosis [43], is an inducer of epithelial-to-mesenchymal transition (EMT) in cancer [6,44], and is also a suppressor at early and promoter at later stages of pancreatic and gastric cancer [5,45–47]. So, targeting this pathway in disease and therapy, and exploiting this pathway in cell-based therapy of the future, is relevant but announces difficult. A strong merger of signaling studies in the developing embryo and stem cells will be needed to make this a success in the future.

Studies of the effects of inhibition of TGFβ signaling in cell culture are the first attempts to document the multiple functions of mainly TGFβ itself. Later, this was also applied to BMPs, including using their secreted inhibitory modulators. The Hoffmann group, in a paper [48] on the successful use of peptide aptamers, derived from the Smad-binding domain of the endosomal associated SIP Sara (Smad anchor of receptor activation; [49]) to interfere with Smad function and its target genes in transplanted cells, summarized these experimental approaches. These included blocking TGFβ synthesis with antisense, and peptide inhibitors that bind to TGFβ or through sequestering it using neutralizing antibodies or (as with BMPs) ligand-binding proteins. TGFβ receptors have been targeted by using neutralizing anti-receptor antibodies, dominant-negative forms of receptors or of Smad2/Smad3, which bind to the type I receptor of TGFβ liganded complex but cannot be phosphorylated. Small-molecule compounds that inhibit serine/threonine kinase domain within the receptors (SB431542)[50–52], and more recently for BMP receptors (dorsomorphin), have been identified [53].

1.3. TGFβ as critical regulator of stem cell functions

Fascinating insight is being gained in the stem cell field from studies on the self-renewal, maintenance and gradual loss of pluripotency, and differentiation of cells, and the role of growth factors herein. Examples of studied cell types include mouse and human embryonic stem cells (ESCs) as well as other embryonic (mouse epiblast-derived cells, EpiESCs; amniotic epithelial stem cells, AECs; neural crest stem cells, NCSCs) or adult cells with stem cell-like properties, e.g. multi-potent adult progenitor cells (MAPCs), mesangioblast/vessel-associated adult multi-potent stem cells (Mabs), neurogenic cells in the dentate gyrus and the subventricular zone of the lateral ventricle in adult rodent brain (NPCs), and hematopoietic stem cells (HSCs). Other breakthroughs were recently achieved in the re-programming of differentiated cells into pluripotent cells (iPS cells, induced pluripotent cells) by nuclear transfer, but more recently by transfection of vectors or viral transduction encoding appropriate combinations of key transcription factors (TFs) or by chemicals [54–60].

Several extrinsic cytokines/growth factors regulate the genes encoding key TFs or differentiation signals that regulate stem cell properties, but it is in many cases still unclear how these signals precisely link to the core circuitry of TFs regulating these properties. In mouse ESCs, the pluripotency-associated TFs consist of Oct3/4 (also called Pou5f1, a home-box-containing factor) and Sox2 (an Sry-related HMG box protein), which bind together at many sites in the genome, and Nanog. These TFs and their partners/co-factors, and hence their target genes, keep the cells from differentiation [61]. The level of Nanog seems to correlate with the degree of pluripotency of a stem cell. It can fluctuate slowly and randomly – thereby giving rise to heterogeneous cell populations – essentially between two states, i.e. stable-high and unstable-low levels, where according to recent mathematical modeling the excitation of Nanog can be driven by transcriptional noise [62], which makes stem cells also interesting cell types to study stochasticity in gene transcription [63].

The key cytokine involved in maintenance of mouse ESCs is Leukemia Inhibitory Factor (LIF), a member of the Interleukin-6
family that signal via gp130 receptor-containing complexes and control signaling by the Jak-Stat pathway. In mouse ESCs, Klf4, another candidate core pluripotency TF, was also found to be mainly activated by Jak-Stat3 leading to Sox2 activation. A parallel circuit involves activation of one of the T-box family members, Tbx3. Its level is preferentially regulated in these cells by the PI3K-Akt and MAPK pathways, leading predominantly to stimulation of Nanog transcription. Overproduction of Nanog supports LIF-independent self-renewal of mouse ESCs by shortcircuiting the need for Klf4 and Tbx3 activity, while forced production of Klf4 or Tbx3 is sufficient to maintain pluripotency and obtain expression of Oct4/4 in the absence of LIF. Klf4 (as well as Tbx3) is thus involved in mediating LIF signaling to the core circuitry but is not directly associated with the maintenance of pluripotency of ESCs in the context studied here [64]. LIF alone however is clearly insufficient to block neural differentiation and maintain pluripotency of mouse ESCs. So, the system requires additional serum-derived factors that promote sustained self-renewal [65]. Furthermore, LIF is not necessary for the maintenance of human ESCs [66], and LIF signaling is not active in undifferentiated human ESCs [66,67].

TGFβ family ligands play an important role in ESC self-renewal, maintenance of pluripotency and regulation of differentiation. In combination with LIF/Jak-Stat3 signaling, low amounts of BMP4, via Smad-mediated activation of Id (inhibitor of differentiation) genes, are compatible with and sufficient to maintain mouse ESC self-renewal in the absence of feeder cells and serum [65]. Additional factors that contribute to the maintenance of pluripotency of both human and mouse ESCs include Wnt proteins. Wnt3a activation sustains the synthesis of the pluripotent stage-specific transcription factors Oct4 and Nanog [68,69]. Oct4 is needed for maintaining expression, chromatin occupancy and higher-order chromatin structure of a locus encompassing the Nanog gene [70]. Thus, it is evident that in addition to LIF other growth factors are involved in the maintenance of ESC pluripotency [71]. Unraveling the relation of these factors with the intrinsic regulators such as Tfs and, in the case of TGFβ signaling, SIPs that are Tfs (e.g. [54,61,72–75]) will not only contribute to a better understanding of the regulation of ESC maintenance, but also their differentiation, depending on the Smad-SIP pathway being studied.

In this review paper, we concentrate on the activities of Nodal/Activin and BMPs, preferably via Smad canonical signaling, identified on selected types of embryonic stem cell and iPS cells, with focus on their pluripotency, plasticity and neural–ectodermal conversion. We do not discuss Smad signaling in embryonic and adult hematopoiesis because excellent reviews on this topic were published recently [76–78].

2. Canonical TGFβ signaling in embryonic stem cells

2.1. Recent important insight from studies in non-mammalian embryos

Breakthroughs in TGFβ family signaling and Smad function in stem cells, including of the early embryo, do not only come from work with mammalian embryos and ESCs.

Mesoderm in Xenopus is induced in the overlying presumptive ectoderm by the zygotic Nodal proteins Xnr1, 2 and 4 [79]. This requires FGFR on top of Nodal activity and the resulting phosphorylation of Smad2 but also of p53 is crucial for mesoderm formation. The mesoderm is then patterned by activated Smad2 at the dorsal side and activated Smad1 in the rest of the mesoderm. This is achieved through carefully regulating BMP activity in the mesoderm. BMP transcripts are first ubiquitously expressed but become rapidly restricted to the lateroventral marginal zone, where the resulting BMP activity is then controlled by secreted modulators of BMP by the Spemann organizer [80]. Importantly, part of the Xenopus ectoderm must succeed in maintaining its pluripotency. In an early phase, this is achieved by the RING finger protein ectodermin, also named Transcriptional Intermediary Factor (TIF)–γ (moonshine in the zebrafish), which blocks overall Smad activity in the ectoderm through ubiquitination of the common-mediator Smad Smad4. This attenuates both Nodal and BMP signaling in this part of the embryo. Subsequently XF DL, a novel partner of p53, blocks p53 and therefore also Smad2, which prevents formation of mesoderm in this part of the embryo [81] (see also comment in Ref. [82]).

Neural induction divides the remaining ectoderm in non-neural and neural ectoderm. The formation of ectoderm is promoted by BMP, whereas that of neural tissue is inhibited by excess BMP and likely promoted in part by the neural-inducing Xnr3 [79]. Endogenous BMP, similarly to what occurs in mesoderm, is neutralized in the neural ectoderm of the embryo through secreted BMP-binding proteins like Chordin, Noggin and Follistatin. Recent fate mapping in the amphibian embryo shows that at the border between these two tissues NCSCs are specified, likely through the combined action of Wnt signals and – again – precise dosages of BMP activity [83,84]. It is not known whether the neuroectoderm of Xenopus in addition to secreted BMP-modulating proteins has an intracellular back-up system consisting of SIP-TFs, which might act in some cases as part of chromatin remodeling complexes [85], and that prevent any residual activity of BMP-Smads.

2.2. Maintenance and generation of pluripotency

ESCs are cultured pluripotent cells derived from epiblast cells of the inner cell mass of the blastocyst. Mouse ESCs are pluripotent as they generate all somatic and germ line cell types in the developing embryo of chimeras. In the mouse, this can also be shown by tetraploid complementation, whereas the pluripotent nature of hESCs is demonstrated as the ability of hESCs to generate teratomas in vivo [86].

Pluripotent mouse cell lines that can be expanded in human ESC culture media, and which are supplemented with FGF2 and Activin, have been derived from the epiblast layer of early post-implantation, pre-gastrula mouse embryos [87–89]. Such iPSCs also express key pluripotency genes such as Oct4, Sox2 and Nanog. Due to epigenetic regulation iPSiSCs express low amounts of Stella [90], a pluripotency marker that is highly expressed in ESCs but is also a marker for primordial germ cells (PGCs). The generation of PGCs is dependent on BMP and Smad1/5 signaling in the mouse embryo [91–95]. It is not yet clear whether Smads function in direct transcriptional regulation of Stella.

Recently, ESC-like cells have been generated from adult somatic cells, named iPS cells (iPSCs). These can be generated from terminally differentiated cells by introduction of four Tfs (Oct4, Sox2, Klf4 and cMyC), causing de-differentiation of the cells into cells with nearly all features of ESCs [96]. Subsequent improvements of this approach, based on the same seminal concept and similar protocols, have yielded human and mouse iPSCs, which have apparently all characteristics of ESCs, including the ability to generate all tissues by tetraploid complementation (mouse iPSCs) and teratoma formation (mouse and human iPSCs) [97]. iPSCs display an epigenetic make-up (i.e. genomic profiles of DNA methylation and histone acetylation and methylation) very similar to that of ESCs [97]. iPSCs have meanwhile been isolated from multiple cell types of human, mouse and rat.

2.2.1. Mouse and human ESCs

At present, the data on TGFβ signaling in maintenance of pluripotency still come mainly from work in mouse and human ESCs. The self-renewal promoting activity of animal serum on
mouse ESCs could be ascribed to BMP2, BMP4 and GDF6 [65,98]. In the absence of these BMP subgroup TGFβ ligands, LIF alone cannot prevent the neural differentiation of ESCs [65]. In the presence of LIF, the specification of early-mesoderm progenitors is reverted to generate fully pluripotent ESCs by a mechanism involving the TFs Nanog and T, prompting the possibility that T regulates Nanog expression in these progenitors [98]. Indeed, not only Stat3 but also T coordinately bind to a regulatory element in the mouse Nanog promoter, resulting in increased Nanog expression in early-mesoderm progenitors. Nanog on its turn prevents the BMP-induced lineage progression into mesoderm by directly binding to BMP-Smads (shown for Smad3 [99]) and interfering with the recruitment of co-activators of the latter. Doing so, transcriptional activation of downstream targets is blocked, including of T itself. It is intriguing that in Xenopus the T homolog Brachyury (Xbra) has been shown to interact with Smad1 also, which is needed for Xbra for directly inducing Xom, encoding a repressor of goosecoid (gsc) that acts downstream of BMP signaling, thereby preventing Xbra from inducing gsc in the mesoderm [100]. This raises also the possibility that individual T family members, some of which are medically relevant, may not only share partners but also have different partners for selectively regulating Smad-regulated processes. However, such partners still remain to be identified and have investigated in LIF-BMP controlled Nanog expression in mouse ESCs.

Human ESCs differ in their requirement for growth factors for their in vitro culture. They do not require LIF, and routine feeder-free cultures need to be supplemented with FGF2 and Activin [101–103]. In these conditions, activated Smad2/3 proteins bind directly to a proximal region of the human Nanog promoter. This effect is sensitive to SB431542 and thus Alk-receptor (likely Alk4) dependent [104]. Similar to mouse ESCs [64], the overexpression of Nanog in human ESCs shortcuts the need for inclusion of Activin (and of FGF2) in the growth medium. Vallier et al. [105] have carefully compared the effects of Activin/Nodal on human ESCs and mouse Episcs. They provide biochemical evidence that Nanog interacts directly with Smad2 and Smad3 proteins and that Nanog is controlled by Activin/Nodal in both cell types.

In human ESCs, unlike in mouse ESCs, BMPs promote via activation of BMP-Smads the formation of trophoderm and thus repress the expression of pluripotency genes, including direct repression of the Nanog promoter by Smad1 [104]. Thus the BMP-Smad pathway functions clearly differently between human and mouse ESCs [98,99,73]. It cannot be excluded that the Activin-Smads and the BMP-Smads, possibly by binding to the same DNA-binding SIP (or perhaps more than one SIP), regulate Nanog expression via the same promoter element in human ESCs, which would suggest that such SIP provides a balancer function between these two fate signals via regulation of Nanog (and possibly of other shared target genes). In such case, one will need to firmly establish whether these transcriptional regulations are dependent on the intact Smad-binding domain(s) (SBD) of the SIP that is involved. In line with this, and considering the observation that Smad1 binds directly to mouse Nanog, it would be interesting to document Activin-Smad and BMP-Smad binding to human Nanog as well, and furthermore whether this is critical for and influences on its turn transcription of a selective set of target genes for Nanog in an SBD-dependent manner.

2.2.2. Amniotic cells

To survive and develop in utero, the mammalian embryo proper develops extraembryonic tissues, but the cells from yolk sac, amnion and possibly visceral endoderm can also provide stem cell-like cells with therapeutic potential [106]. Amniotic epithelial cells (AECs) have been considered a source of stem or stem cell-like cells and should more systematically be included in studies of growth factor signaling. Fairly little attention has been attributed to the early development of amnion, the innermost extraembryonic tissue that surrounds the fetus. In addition to its normal function during in utero development, the amnion can have important clinical applications. It has been used as a dressing to stimulate/improve epithelial wound healing in burns and ocular wounds and was recently proposed for use in treatment of stroke [107,108]. Cells derived from term human amniotic epithelium, and amniotic fluid cells, have stem-cell-like features. 10–20% of human AECs display certain pluripotency markers, e.g. Oct4, Sox2, TRA1-60, TRA1-81, SSEA3/4 [109–114]. It is however still questionable if they are bona fide stem cells, but they are able to differentiate in endodermal but also ectodermal and mesodermal cells [112,115,116]. Since fetal membranes are normally discarded at birth, amnion cells are readily available in large amounts, which make them an attractive source of stem cells. This is in contrast to the small numbers of human adult and ESCs that can be isolated by labor-consuming isolation protocols.

It is still unclear whether AECs are subject to TGFβ family ligand control in vivo as systematic studies in knockout mice for components of the system in this part of the embryo are lacking. An important observation in Smad5 knockout mice strongly suggests a function for this BMP-Smad, and hence BMP signaling, in the amnion. In the absence of Smad5 the amnion differentiates and stem cell markers become expressed ectopically in the amnion at mid-gestation, which is a unique extra-embryonal phenotype amongst all knockout mouse embryos for other components of the TGFβ system. Removal of Smad5 results in an anteriorly located heterogeneous amiocyte population with stem-cell-like features [95], suggesting that this BMP-Smad is crucial for maintaining amnion homeostasis.

2.3. Selected examples of differentiation

2.3.1. Neural conversion of ESCs and iPS cells

The neural differentiation of mouse ESCs in culture is similar to that observed in the embryo as it involves inhibition of BMP signaling in the ectoderm [117–120]. Hence, treatment of ESCs with Noggin has been used in several neural induction protocols. Neurons have been generated first by the co-cultivation of ESCs with stromal cells in serum-free medium (e.g. [121]). However, co-culture or multi-cellular aggregation in embryoid bodies is not required for ESCs to commit efficiently towards a neural fate. Indeed, elegant monodherent cell culture protocols (e.g. [122]) involve the elimination of inductive signals (withdrawal of LIF and serum) and the addition of certain supplements. By day 4 of culture most of the mouse ESCs form in an asynchronous manner typical rosettes of Sox1-positive neuro-epithelial precursor cells, which loose Oct4 expression and become nestin-positive. While undifferentiated ESCs (10–15% of the total) persist in the cultures, the nestin-positive neuroblasts then rapidly develop into neurons, followed by astrocytes and oligodendrocytes. Very few non-neural, e.g. meso/endodermal cells are present in these cultures. This culture system also critically requires FGF signaling in the very early phase. Very low levels of endogenously produced BMP can be detected, but endogenous Noggin and Follistatin have been proposed to neutralize it. The addition of extra Noggin does not promote neural conversion, whereas addition of 10 ng BMP/ml, which is a low concentration of BMPs compared to the amounts (50–100 ng/ml) used in other cell culture based bio-assays, suppresses it.

In human ESCs, Nodal was found to inhibit differentiation along the neuroectodermal lineage. This was initially concluded from experiments involving overexpression in ESCs of Lefty2 and a truncated form of Cerberus (Cer-S) that only retains Nodal-binding activity but no longer Wnt and BMP binding [123]. Embryoid
bodies derived from either Lefty or Cer-S overexpressing human ESCs showed increased levels of Sox1 and Nestin expression, failed to generate definitive endoderm (Sox17) and beating cardiomyocytes, i.e., cell types that have been shown to be promoted by Nodal (see Ref. [124]). After plating the embryoid bodies, the cultures formed significantly higher numbers of neurons as compared to wild-type human ESCs [125]. In the same study, treatment of wild-type human ESCs with SB431542 gave the same results. Thus, inhibition of Nodal signaling promotes specification of neurons in human ESCs.

An elegant next refinement of neural conversion of human ESCs was possible by combining the two findings discussed above. Indeed, Chambers et al. [126] recently reported the establishment of a high-efficiency neural conversion protocol under (mono-)adherent conditions by exposing the cells to a combination of Noggin and SB431542 during 11–13 days. Further investigation of the mechanisms underlying their clear synergistic action, in addition to the destabilization of the Activin pluripotency signaling, indicates that this place action may also take place at multiple stages of differentiation, including a rapid loss of Nanog expression (loss of pluripotency). Noggin represses Cdx2 expression, which otherwise would increase upon exposure with SB431542 only, indicating the role of Noggin in the suppression of trophoblast fate by endogenous BMP. The highly efficient neural conversion can possibly also be explained by suppression of endodermal fate (Sox17) and by SB431542-mediated mesendodermal fate (Bra, T). So, mesendodermal fate is suppressed by inhibiting endogenous Activin/Nodal, which coincides with the promotion of neuralization of primitive ectoderm/epiblast through inhibition of BMP action. Monitoring of the lineage progression of these treated human ESCs indicated the formation of epiblast-stage cells (Oct4-negative, Otx2 and Fgf5-positive) prior to neuralization. Surprisingly Sox1 appears first, followed by the neuro-epithelial markers Zic1 and Pax6, which suggests that Sox1 is normally under control of Smad signaling in untreated cells. This is followed by anterior neural cells (central nervous system, FoxG1) and NCSCs (p75, HNK-1) (at low cell density of plating at the onset of the experiment) or motor neurons and dopaminergic neurons (at high density). This illustrates a robust patterning response in these treated cells.

Chambers et al. [126] then tested if this dual-Smad inhibition protocol could be used on iPSCs. In their published work, they demonstrate this with iPSC cells derived from fetal lung fibroblasts after viral transduction of CMyC, Klf4, Oct4 and Sox2 cDNA. Neural crest progeny and specific neuron subtypes were efficiently generated using the dual-inhibition protocol and this in a shorter time than in differentiation protocols that require stromal feeder cells.

### 2.3.2. Mesodermal and endodermal conversion of ESCs

ESCs can also differentiate into cells of the mesoderm lineage, e.g., skeletal and cardiac muscle [127,128]. Myogenesis (for reviews, see Refs. [129,130]) involves many signaling pathways including Shh and Wnt1/3a. Shh plays a key role in epaxial myotome formation by regulating Myf5, while Wnt1/3a activates the canonical β-catenin pathway, which leads to the induction of Myf5 and MyoD [131]. BMP4 and BMP7 generally ventralize mesoderm. Somites express Noggin and Follistatin to inhibit the ventralizing action of BMP4 and BMP7 for a review, see Ref. [132]). Nevertheless, BMP4 and BMP7 induce Pax3 leading to proliferation of myogenic precursors such that sufficient precursors are generated for the muscles of the body [133]. Pax3 is an early marker gene for skeletal muscle specification and is expressed in pre-somatic mesoderm and in early somites. It is important for migration of cells from the dermamyotome and proliferation of myogenic precursor cells. Pax7, detected in the maturing somite with Pax3, is not essential for myogenic specification but is needed to form satellite cells [134,135]. The basic helix-loop-helix TFs MyoD and Myf5 are important for muscle determination, as their combined removal in mice causes perinatal death due to a lack of skeletal myoblasts, while homozygous single knockouts do not display a myogenesis phenotype. Myogenin is in the mouse necessary for myofiber formation as knockout mice generate myoblasts but not myofibers. Myf6 combined with Myf5 activates MyoD expression. Myf6 is also involved in myofiber formation, though to a lesser extent than myogenin.

To induce mouse ESCs to the myogenic lineage a combined stepwise induction by cytokotines (Activin-A and Wnt3) is used first to induce primitive streak, followed by combinations of BMPs and FGFs to induce mesoderm, then inhibition of TGFβ signaling with addition of FGFs, IGF and HGF to induce the myogenic lineage, and then selection for cell surface antigens on mesoderm (Fk1/PDGF) myogenic committed (CD34/NCAM) cells and/or selection based on the presence of Pax3. ESC differentiation to cardiomyocytes has been done using embryoid body formation, e.g., by selecting subpopulations of Fk1+CXCR4+ mesoderm committed cells [136,137] from monolayer cultures treated with 10 ng Activin/ml or from embryoid bodies treated with 10 ng Activin/ml that are then allowed to re-aggregate before plating on gelatin [138]. Cardiomyocytes have been obtained also in monodherent cell cultures wherein ESCs were cultured without serum, but with 100 ng Activin/ml for 24 h followed by 10 ng BMP4/ml [138], Nkx2.5, GATA4-6, Tbx5 and Tbx20 are the key TFs for cardiac determination as well as serum response factor (SRF). SRF plays a determinant role in cardiogenesis by driving the expression of silencer miRNA and regulating the biogenesis of muscle contractile proteins.

Development of mature hepatocytes from ESCs, as an example of endodermal conversion, can grossly be divided into four sequential steps. During gastrulation primitive ectoderm cells migrate under the influence of Nodal/Cripto and Wnt towards the primitive streak, where they ingress [139,140] to form mesendoderm and subsequently definitive endoderm (DE) [141] This was mimicked in vitro by exposing the cells for 6 days to 100 ng Activin-A/ml (to replace the Nodal/Cripto signal) and 50 ng Wnt3a/ml. Subsequently, DE is specified towards a hepatic fate by cytokotines secreted _in vivo_ by the adjacent cardiac mesoderm and septum transversum mesenchyme, which was mimicked in vitro by adding 10 ng FGF2/ml and 50 ng BMP4/ml [142–144]. Afterwards hepatoblasts proliferate and start to mature further, which is governed _in vivo_ by precise concentrations of FGFs. Final maturation guides hepatoblasts towards a hepatic fate, which can be accomplished by the addition of 20 ng HGF/ml, a general hepatotrophic cytokine [145] and 100 ng Follistatin/ml to block cholangiocyte differentiation [146]. During the entire protocol high concentrations of dexamethasone are added to the differentiation medium.

Although directed differentiation can be obtained via what appear transcriptional–developmental correct steps, the precise signaling mechanisms underlying such differentiation are still unknown. Initial studies demonstrate that addition during step 1 of Activin-A and Wnt3a activates downstream proteins known to be induced by these cytokotines. For instance, Fgf8 is a downstream target of Wnt signaling, and its induction is required for primitive streak formation and migration of cells into the streak [147], whereas Foxh1, combined with Smad2/3/4 regulates Nodal expression and its downstream target genes. Foxh1 is also important for the amplification of Nodal expression before and during gastrulation [148].

### 2.3.3. Canonical TGFβ signaling in adult neurogenesis

One of two acknowledged adult neural stem cell niches, the subependymal zone (SEZ), can generate new neurons in the central
nervous system [149–151]. Astroglia in the SEZ act as stem cells in these niches and generate transit-amplifying precursor cells that then give rise to neuroblasts and eventually neurons of the olfactory bulb. The adult SEZ stem cell niche (containing type B cells, the astrocytes of the subventricular zone (SVZ) and representing the GFAP, Vimentin and Nestin-positive (+) stem cells) generates Dlx2+ Nestin+ transit-amplifying precursor cells (TAP, also named C cells) that give rise to neuroblasts (A cells, NCAM+, Dlx2+, Tuj1+). In the case of the SEZ, these neuroblasts proliferate mainly in the SVZ, and then migrate along the rostral migratory stream (RMS) to reach the olfactory bulb (OB), where they migrate radially and undergo terminal differentiation into neurons. This adult neural stem cell niche forms a unique environment for neurogenesis, but in the SEZ little is known about the extrinsic factors promoting adult neurogenesis as compared with intrinsic fate determinants (e.g. the TFs Pax6 and Mash1 in neurogenesis, Olig2 in adult oligodendrogligenosis) [152–154].

BMP signaling inhibits neurogenesis, and secretion of Noggin from the ependymal cells is crucial to allow neurogenesis in the SEZ [155]. The group of Götz [42] recently studied TGFβ family signaling in the adult SEZ by inducible conditional ablation of the Smad4 gene in neural stem cells of adult mice. Smad4 gene ablation severely impairs neurogenesis and reduces Psmad1/5/8 levels. They concluded that the cell-autonomous functions of Smad4 in the interpretation of BMP signaling are impaired and also showed that the BMP pathway is active and required in adult SEZ stem cells to allow neurogenesis and inhibit oligodendrogligenosis. However, overexpression of a BMP receptor Alk3 in the post-natal SEZ, carried out by the same team, does not affect neurogenesis. In our opinion, this important study convincingly paves the road for new studies of BMP signaling in adult neurogenesis, including the intrinsic control by BMP-SmadS and their SIPs present in these stem cells and/or their progeny.

3. Conclusion and future needs

We decided to focus in this review on a limited number of aspects of Activin/Nodal and BMP ligand and Smad signaling in various types of stem cells, with the main focus on ESCs, for it would be impossible to include more information on – for example – BMP function in NCCs and their progeny and the peripheral nervous system, as well as neuron and non-neuronal subtype differentiation in the central nervous system (e.g. [156]) and above all the vast information resulting from in-depth analysis of knockout mouse models and ex vivo experiments with cells derived from these.

Despite the optimistic predictions with regard to improved tissue regeneration, the derivation of fully functional therapeutic cells from stem/progenitor cells that are induced to differentiate in a controlled manner ex vivo and in vivo, amongst others by growth factors, and that undergo appropriate epigenetic regulation as well, appears much more complex than previously expected (for reviews, see Refs. [157,158]). It seems that instructions to stem/progenitor cell cultures in an attempt to mimic what happens in vivo during normal development can still only be recreated in part, thereby preventing further progress of the field. Indeed, it is difficult to mimic in growth factor supplemented cell culture or in current bio-artificial tissues the spatial organization of cells and their lineage progression and stepwise differentiation under the determining influence of various types of cell–cell and cell–matrix interaction. In order to close the gap between the in vitro or ex vivo stem cell systems and the in vivo conditions, there is a need to study the regulation of cell renewal, pluripotency and differentiation in the early embryo in extreme depth and test these findings in stem cell culture systems. This explains why so many stem cell biologists, embryologists and ideally also signal transduction researchers are teaming-up and brought together in many institutes in the first place.

Specifically with regard to TGFβ family signaling, it is clear that these studies have already started and still continue primarily with the careful documentation of the effects of selected key ligands of this family, i.e. TGFβ, but mainly Nodal/Activin and BMPs and some of their secreted modulators, on stem and progenitor cells [159,160]. Fewer studies have yet addressed the functions of specific Smads in stem cells. In addition, significant control is achieved through making the Smads corrodorate with SIPs (e.g. Tfs), and Smads and/or SIPs with chromatin-modifying/remodeling complexes, to regulate the expression of target genes, thereby forming the endpoint of a cell-type specific signaling event after exposure of cells to TGFβ family ligands. Therefore, the field will have to move into the analysis of the multiple functions of these SIPs, which furthermore and especially in the case of multi-domain SIP-TFs may not depend all on Smad interaction and hence will have to be carefully designed and their results interpreted with caution. In our opinion, there is an urgent need to expand the experimental approaches in various stem cells with the meticulous analysis of the various spatial–temporal controls that underlie the versatile actions of receptor-regulated Smads with many of their non-Smad SIPs, and to identify their direct target genes resulting from their individual and co-operative action, respectively.

In the entire embryo or in its tissues/organs and in ESCs, and in other stem/progenitor cells in their embryonic niche, evidence is emerging that the expression of the different components of the TGFβ family signaling system is linked by synexpression and feedback control mechanisms. However, a much needed systems-biology type of overview of these relationships between all known components, including in quantitative terms, is still lacking even in non-stem cells or in selected embryonic regions. In a recent study, mouse ESCs have been used in which levels of activated Smad2/3 could be specifically upregulated or downregulated. Analysis of the transcriptome of these cells revealed that the level of activated Smad2/3 is converted proportionately in the ESC transcriptional response. Genes encoding positive and negative regulatory proteins of TGFβ signal transduction were amongst the identified direct target genes. Importantly, the activation of genes encoding negative intracellular feedback regulators, which interfere with phospho-Smad2/3 transcriptional activity, did not have a major effect on the changes resulting from Smad2/3 modulation over quite some time [161]. It remains to be shown whether this also holds in the early embryo, where such feedback, mainly by secreted proteins, has clearly been documented for limited numbers of components in single gene knockout/knockdown experiments in embryos. Target genes of the TGFβ-Smad family system comprise genes encoding components of the TGFβ system, revealing synexpression, complex autoregulation and feedback signaling control within the TGFβ system. In-depth study of the signaling network between progenitor and differentiated cells or between stem cells and their niche, will provide deeper and essential knowledge of these network features in qualitative and quantitative terms (as approached in Ref. [162]).

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An Zwijsen is VB1 group leader in the Department of Molecular and Developmental Genetics (VIB11) and the Center for Human Genetics, University of Leuven, Belgium. She received her Ph.D. in 1996 in the group of V. P. Van Leuven, Center for Human Genetics, Leuven and continued there with her post-doc to study the function of several proteins, using ES cells and knockout mice. She joined Danny Huylenbroeck in 1999, where her research mainly focuses on the role of Smads in angiogenesis in the vertebrate embryo, and she is also involved in many other mouse and ES-cell projects on Smad-interacting proteins.

Eve Stenfjell is pharmacist (1994) and obtained her Ph.D. degree (1999) with a study of cell lineage determination in the pituitary of the mouse embryo. As post-doc, she joined the M. Treier lab at the European Molecular Biology Laboratory, Heidelberg, Germany to study Notch signaling in the pituitary and the role of the homeodomain factor Bsx in hypothalamus development. She is currently senior post-doc in the laboratory of Danny Huylenbroeck (Leuven, Belgium) where she and her group are investigating several proteins, using ES cells and knockout mice. She joined the lab of Danny Huylenbroeck in 1999, where her research mainly focuses on the role of Smads in angiogenesis and the vertebrate embryo, and she is also involved in many other mouse and ES-cell projects on Smad-interacting proteins.
Catherine Verfaillie (MD, University Leuven, 1982) was resident from 1982 to 85 and then fellow in Hematology in Leuven till 1987. She then moved to the University of Minnesota, Minneapolis, MN, USA, where she became Assistant Professor (1991), Associate Professor (1995), and Professor (from 1998). She has been the head of the Stem Cell Biology Program there since 1996 and then director of the Stem Cell Institute. Since 2005, she is Professor of Medicine and the director of the Stem Cell Institute at the University of Leuven. Her research studies the cellular and molecular properties of normal hematopoietic stem cells and also of pluripotent stem cells in comparison with embryonic stem cells, their lineage commitment and evaluation of their therapeutic potential in animal models of congenital disorders.

Danny Huylebroeck is Professor of Developmental Biology, at the Faculty of Medicine of the University of Leuven, Belgium, and VIB group leader there of the Laboratory of Molecular Biology in the Department of Molecular and Developmental Genetics (VIB11) and the Center for Human Genetics. From 1995 till 2007 he was director of the smaller Department of Developmental Biology of VIB on the Leuven campus. He received his Ph.D. from the University of Gent (1985) through research on antigenic drift and shift of influenza viruses in the Laboratory of Molecular Biology (with W. Fiers). Thereafter, he was a 2-year EMBO post-doc fellow in the Cell Biology Program at the European Molecular Biology Laboratory, Heidelberg, Germany, in the group of H. Garoff. Since 1988, he studies where and how various components of the TGFβ signaling system determine cell fate and differentiation in vertebrate embryos.