Wnt/β-catenin's regulatory role in embryonic stem cell pluripotency and differentiation

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Abstract

Stem cells need to tightly regulate the switch between self-renewal and differentiation. Several external signaling pathways contribute to this regulation, together with the internal regulatory gene network. One of the signaling pathways that promotes embryonic stem (ES) cell self-renewal is the Wnt/β-catenin pathway. However, Wnt signaling is also known to induce differentiation of ES cells. This raises the question of how it is possible that the same signaling pathway promotes both pluripotency and differentiation and how the Wnt pathway cooperates with the internal transcriptional circuitry to regulate these processes. Here, an attempt will be made to address these questions; this thesis will discuss the different reports that show a role of Wnt signaling in promoting either differentiation or pluripotency. I will describe how the Wnt/β-catenin pathway interplays with the regulatory gene network of pluripotency in ES cells and finally I will try to explain how it is possible that Wnt signaling plays two distinct roles in the regulation of these processes. There have been several hypotheses proposed of how the Wnt/β-catenin pathway promotes pluripotency. The first suggests that β-catenin directly regulates the internal regulatory gene network of pluripotency in ES cells, which consists of OCT4, SOX2 and NANOG. TCF3 acts as repressor of this regulatory circuitry and β-catenin can relieve this repression, promoting ES cell pluripotency. The second hypothesizes that a direct interaction between OCT4 and β-catenin promotes pluripotency as well. The last hypothesis proposes a role for β-catenin/TCF mediated transcription in maintaining ES cell pluripotency. Aside from promoting pluripotency, Wnt/β-catenin signaling has been shown to induce differentiation. Whether Wnt/β-catenin signaling promotes ES cell pluripotency or differentiation might be a result of differential co-activator usage. Two histone acetyltransferases CBP and p300 could act as co-activators of β-catenin mediated transcription; CBP together with β-catenin promotes pluripotency, whereas p300 as a co-activator induces differentiation. Other external signaling pathways may determine the choice of co-activator usage and hence influence the outcome of Wnt/β-catenin signaling.

Contents

Chapter 1 Introduction

Stem cells are characterized by their ability to both self-renew and to differentiate into different cell-types and often categorized by their developmental potential. For example, a totipotent stem cell has the potential to form all lineages of an organism, including extra-embryonic tissue. The only totipotent cells in mammals are the zygote and early blastomeres. Pluripotent stem cells are able to form all the cell types of the body (Solter, 2006). An example of pluripotent stem cells are embryonic stem (ES) cells. These ES cells have the capacity to self-renew indefinitely not only *in vivo* but also *in vitro*. With the use of different culture conditions, ES cells can be maintained in a pluripotent state or can be guided to differentiate into specific cell fates.

Recently there is a lot of interest in ES cells. This interest has been raised by the research possibilities and clinical potential of these cells; ES cells can be used to study developmental processes and processes that are involved in the self-renewal of ES and cancer cells. Alternatively, ES cells can be used in drug screens and in regenerative medicine. Since ES cells are able to form all the different tissues present in the human body, theoretically ES cells can be used to regenerate tissue and heal patients with diseases where there is no cure available, such as Alzheimer's disease. Perhaps in the future ES cells may even be used to regenerate (parts of) organs. A disadvantage of the use of common ES cells to regenerate tissue is that the patient's immune system will reject the transplanted tissue, as these cells are not isolated from the patient's body. Ideally, pluripotent stem cells that are able to form all different tissues from the patient will be used to regenerate damaged tissue, but such stem cells are not present in the adult human body. However, recent finding have shown that fully differentiated cells can be reprogrammed to a pluripotent state.

Despite the great research- and clinical potential of ES cells, there has been a lot of debate about the use of these cells. This is mainly caused by the fact that ES cells are extracted from unborn embryos. This objection can be overcome by using another source of pluripotent cells. Takahashi and Yamanaka found that fibroblasts can be reprogrammed into a pluripotent state (Takahashi and Yamanaka, 2006), a discovery that earned them the Nobel price of medicine in 2012. When adult cells are reprogrammed to a pluripotent state, these induced pluripotent stem (iPS) cells could replace ES cells in several applications. As an exciting possibility, tissue from patients could be regenerated with iPS cells derived from their own bodies. This would prevent rejection of the tissue by the patient's immune system and eliminate the need for embryos during the procedure. Although this is an exciting prospect, currently it is not yet feasible. It needs to be determined if iPS cells are indeed equal to ES cells and the molecular regulation of this pluripotency needs to be understood to be able to fully exploit the potential of ES and iPS cells.

In order to culture and use ES and iPS cells, specific culture conditions are needed to maintain their pluripotency or to promote the cells to differentiate into specific lineages (reviewed by Ohtsuka and Dalton, 2008). Certain ligands promote pluripotency, while others induce differentiation (Levenstein et al., 2006; Matsuda et al., 1999; Xu et al., 2002; Ying et al., 2003). Besides external signals, the inherited internal gene regulatory network components are crucial for the maintenance of the pluripotent state as well. Deletion of parts of the core transcriptional network regulating pluripotency leads to loss of pluripotency and subsequent differentiation into extra embryonic lineages (Masui et al., 2007; Mitsui et al., 2003; Nichols et al., 1998). This shows that the balance between maintaining pluripotency and initiating differentiation is highly regulated and dependent on both the internal transcriptional regulatory gene network and external signaling pathways. Understanding of both the internal and external regulation of pluripotency and differentiation of ES and iPS cells is crucial for taking advantage of their therapeutic potential.

The core transcriptional regulatory gene network

The internal regulatory gene network responsible for maintaining pluripotency is conserved among vertebrates and involves specific transcription factors. Four of these factors; Oct4, Sox2, Klf4 and c-Myc were shown to be able to induce pluripotency in differentiated cells when expressed ectopically (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Other factors such as Lin28 and transcription factors Nr5a2, Nanog and Esrrb are able to induce pluripotency as well (Feng et al., 2009; Heng et al., 2010; Yu et al., 2007). This shows that many factors are involved in the regulation of pluripotency. Of all these factors Oct4, Sox2 and Nanog seem to be the most important, as earlier work showed that Oct4 and Sox2 work together with Nanog to form the "core transcriptional regulatory gene network" regulating pluripotency (Boyer et al., 2005), which will be described below.

Oct4 and Nanog were among the first factors shown to be indispensible for the pluripotency of ES cells (Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998), later Sox2 turned out to form heterodimers with Oct4 and to be required for the maintenance of pluripotency of ES cells as well, most likely by regulating the levels of Oct4 (Masui et al., 2007). Together these three transcription factors co-occupy many target genes (Boyer et al., 2005). Among these targets are genes important for the pluripotent state of ES cells - such as ES cell transcription factors, chromatin remodeling factors and specific miRNAs - and they also bind to their own promoters. By binding to their own promoters the core regulatory genes create a regulatory feed forward loop that maintains their own expression and thereby reinforces pluripotency. The core regulatory transcription factors also bind genes that are repressed in ES cells, genes that are important during differentiation in different cell fates. These latter target genes remain poised for induction during differentiation (Boyer et al., 2005).

Knowledge of the core regulatory gene network could provide insight in how the overexpression of components of this regulatory network could lead to induction of pluripotency in differentiated cells. The explanation could be that overexpression of these factors activates endogenous Oct4, Sox2 and Nanog genes, which subsequently stabilize their own expression, activate their targets and thereby reinforce pluripotency. Despite the fact that Oct4, Sox2 and Nanog are important for the internal transcriptional regulation of pluripotency, ES cells cannot maintain pluripotency without other factors or external signals.

External signaling pathways

The external signals that regulate ES cell pluripotency are different for human (h) and mouse (m)ES cells. mES cells utilize three pathways to maintain their pluripotency; the LIF, BMP4 and PI3K pathways. The best known growth factor in mES cell self-renewal is leukemia inhibitory factor (LIF). When LIF is provided to the mES cells it activates STAT3 and destabilize glycogen synthase kinase-3 (GSK3) (Cartwright et al., 2005). Bone morphogenetic protein (BMP)4 can maintain pluripotency of mES cells together with LIF in serum free conditions, by inducing inhibitor of differentiation (Id) genes through the Smad pathway (Ying et al., 2003). Besides LIF and BMP4, phosphatidylinositol 3 kinase (PI3K) signaling activates AKT and is also known for its role in mES cell pluripotency, activating components of the core regulatory circuitry (Paling et al., 2004).

hES cells require completely different external signals to maintain their pluripotency. Fibroblast growth factor (FGF)2 is needed to maintain pluripotent hES cells (Levenstein et al., 2006), probably by activating the core transcriptional gene network (Greber et al., 2007). In cooperation with FGF2, Activin/Nodal signaling plays a role in the maintenance of pluripotency as well (Vallier et al., 2005). Signaling of insulin/insulin-like growth factor (IGF) was also shown to be involved in the pluripotency of hES cells by activating PI3K signaling (McLean et al., 2007). This makes FGF2, Activin/Nodal and insulin/IGF signaling important for the maintenance of hES cells.

LIF was shown to be dispensable for the maintenance of hES cells, in contrast to mES cells (Humphrey et al., 2004). Similarly, BMP4 is not needed to keep hES cells in a pluripotent state, BMP4 even induces hES cells to differentiate into the trophoblasts (Xu et al., 2002). Thus, largely different

pathways seem to be involved in the maintenance of human and mouse ES cells with PI3K signaling as of the few signaling pathways that promotes pluripotency in both systems.

GSK3 might be a key component in the regulation of pluripotency of ES cells by external signals. GSK3 was shown to play a role in both LIF and PI3K signaling (Bechard and Dalton, 2009). One of the mechanisms whereby GSK3 might control pluripotency is by regulating c-Myc stability. c-Myc promotes self-renewal of ES cells (Cartwright et al., 2005). PI3K and LIF signaling inhibit GSK3 activity, allowing c-Myc to be stabilized and promote ES cell self-renewal. PI3K and LIF have different roles in hES cells, despite the fact that they both control GSK3 stability, since LIF is unable to promote pluripotency in hES cells. Besides being involved in the PI3K/AKT and LIF/STAT pathway, GSK3 is also a well known effector for inhibition by Wnt signaling (Aberle et al., 1997; Cook et al., 1996). This suggests that Wnt signaling might be involved in maintenance of pluripotency of ES cells as well. Indeed, both human and mouse ES have been reported to benefit from the activation of Wnt signaling (Pereira et al., 2006; Sato et al., 2004; Singla et al., 2006). This shows that together with PI3K signaling, Wnt might be one of the few common regulators of pluripotency in both human and mouse ES cells.

Wnt signaling was already well established to be involved in somatic stem cell self-renewal (Gregorieff et al., 2005; Reya et al., 2003), before it was found to be involved in the self-renewal of ES cells. There are quite some reports about Wnt signaling promoting ES cell self-renewal (Cai et al., 2007; Ogawa et al., 2006; ten Berge et al., 2011; Wagner et al., 2010), however, there are several other reports that show that Wnt signaling promotes differentiation in ES cells as well (Bakre et al., 2007; Davidson et al., 2012; Lindsley et al., 2006). This shows that the precise role of Wnt signaling in the pluripotency of ES cells is not yet fully understood and still a topic of debate.

This thesis will summarize the findings of Wnt signaling involved in either pluripotency or differentiation in ES cells and will try to show how the pathway might link into the core transcriptional regulatory gene network. In Chapter 2 an overview of the canonical Wnt/β-catenin pathway will be given, to show how the signal is relayed from the membrane through the cytoplasm into the nucleus. In Chapter 3 the findings showing that Wnt signaling is involved in somatic-, embryonic- and induced pluripotent stem cells will be discussed. The fourth chapter will try to elucidate how Wnt signaling might control pluripotency through the core transcriptional regulatory gene network, mainly by either acting directly on Oct4 or to relieve repression by Tcf3. In the fifth chapter the conflicting results showing Wnt signaling being involved in promoting differentiation will be highlighted and discussed. The last chapter will summarize the findings of the thesis.

Chapter 2 Canonical Wnt signaling

The Wnt gene was originally found in mouse breast tumors as a preferential integration site of the mouse mammary tumor virus (Nusse and Varmus, 1982). This gene was first named *int*. When researchers isolated the *Drosophila* homolog of *int,* they found that it was identical to the previously described segment polarity gene *wingless* (*wg*) (Nusslein-Volhard and Wieschaus, 1980; Rijsewijk et al., 1987). The names *int* and *wg* were subsequently put together to create the name Wnt. Combined observations in *Drosophila* and *Xenopus* embryos showed that Wnts are secreted proteins that can activate different signaling cascades (Clevers, 2006).

There are several Wnt signaling pathways that are usually split up into two groups; the canonical pathway, also called the Wnt/β-catenin pathway and the non-canonical pathway. The canonical pathway is the Wnt signaling cascade that, when overexpressed, can induce axis duplication in *Xenopus* embryos. Activation of this pathway stabilizes β-catenin and subsequently activates TCF mediated transcription. The non-canonical pathways are β-catenin independent pathways and are called planar cell polarity (PCP) and Wnt/Ca^{2+} pathway, which control cell polarity and morphogenetic processes respectively (Kohn and Moon, 2005). The canonical and non-canonical pathways have historically always been viewed as separate pathways, but recent insights show that these pathways are more interconnected (reviewed by van Amerongen and Nusse, 2009). This thesis will mainly focus Wnt/β-catenin pathway in (embryonic) stem cells. In the rest of this chapter an overview of the canonical Wnt pathway will be given.

Degradation of β-catenin in the absence of Wnt signals

β-catenin is the main effector of the canonical Wnt cascade. In absence of a Wnt signal, β-catenin is targeted by the destruction complex for degradation by the proteasome (Aberle et al., 1997) (Fig 1A). Axin serves as a scaffold, binding all the other proteins in this destruction complex. It binds the tumor suppressor adenomatous polyposis coli (APC), the protein kinases GSK3 and casein kinase 1 (CK1) and, in absence of Wnt signals, also β-catenin. Conserved residues of the N-terminus of β-catenin are first phosphorylated by CK1 and subsequently by GSK3, when β-catenin binds to the destruction complex (Liu et al., 2002). This phosphorylation is needed for β-TrCP to recognize β-catenin. β-TrCP is part of an E3 ubiquitin ligase complex and ubiquitinates β-catenin. This ubiquitination targets β-catenin for subsequent degradation by the proteasome (Aberle et al., 1997). Apart from its role in Wnt signaling, β-catenin plays a role at the adherens junctions as well. At these adherens junctions, β-catenin binds to E-cadherin (Peifer et al., 1992). This pool of β-catenin bound to the membrane is stable even without Wnt signaling. The current hypothesis is that newly synthesized β-catenin first binds at the adherens junctions and the unbound β-catenin will be targeted by the destruction complex for degradation by the proteasome in the absence of Wnt signals. Upon activation of Wnt signaling is present, this cytosolic pool gets stabilized.

Wnt-dependent stabilization of β-catenin

Wnt signaling starts with secretion of Wnt ligands that are palmitoylated on a conserved cysteine residue. This modification is important for the signaling function of secreted Wnts (Willert et al., 2003). Secretion of Wnt is aided by Wntless/evenness interrupted (Wls/evi) (Banziger et al., 2006; Bartscherer et al., 2006). Upon secretion a Wnt ligand will bind to a member of the family of Frizzled (Fz) receptors, which is a family of seven-pass transmembrane proteins (Bhanot et al., 1996). A co-receptor is needed in addition to Fz to activate Wnt signaling. In the canonical Wnt signaling pathway this typically is the single-pass transmembrane receptor low-density-lipoprotein receptor-related protein (LRP)5 or LRP6 (Pinson et al., 2000; Wehrli et al., 2000). Fz and LRP5/6 are able to bind to each other (Zeng et al., 2008) and both receptors are needed to activate Wnt signaling. This suggests that interaction of Wnt with both Fz and LRP5/6 is needed, although it has never been shown directly that Fz, LRP and Wnt form a complex together.

Upon binding of Wnt to Fz and LRP5/6, Dishevelled (Dvl) is recruited by Fz (Wong et al., 2003). Wnt signaling controls phosphorylation of Dvl (Yanagawa et al., 1995), although the precise mechanism is unknown. Dvl is able to multimerize, which is thought to drive signaling, since it provides the cell with a high local maximum of receptors and possibly increases the affinity with its interaction partners (Schwarz-Romond et al., 2007a). The multimerization of Dvl was shown to be crucial for recruiting Axin as well. LRP5/6 also gets phosphorylated upon Wnt binding, through sequential activity of GSK3 and membrane bound CK1γ (Davidson et al., 2005; Zeng et al., 2005). Phosphorylation of LRP5/6 also promotes binding of Dvl and triggers the recruitment of Axin to the membrane. Membrane recruitment likely triggers a conformational change in Axin (Lee et al., 2003). This conformational chance is expected to lead to either degradation of Axin or to a change in its binding affinity for other components of the destruction complex (Schwarz-Romond et al., 2007b). When the destruction complex is no longer active, β-catenin will not be phosphorylated. This prevents β-catenin recognition by β-TrCP and subsequent degradation by the proteasome. Thus absence of phosphorylation leads to stabilization of β-catenin (fig 1B).

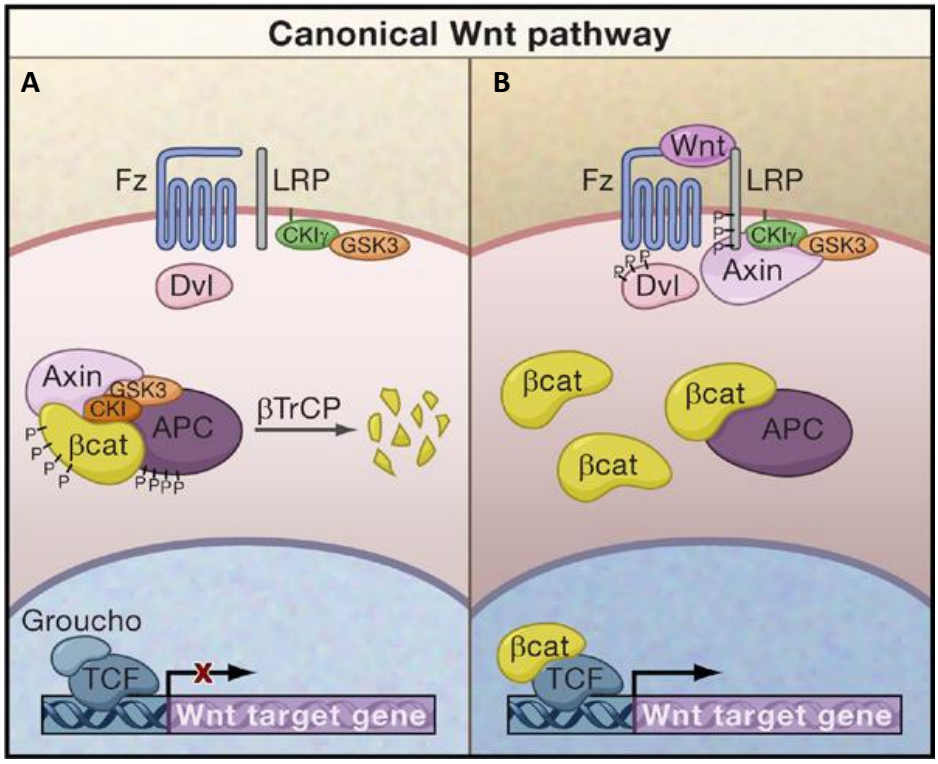

Figure 1 Overview of the canonical Wnt pathway. A) In the absence of Wnt signaling, β-catenin is phosphorylated by the destruction complex consisting of Axin, CK1, GSK3 and APC. β-TrCP recognizes this phosphorylation and ubiquitinates β-catenin, targeting it for destruction by the proteasome**. B)** When Wnt signaling is present, Wnt binds to Fz and LRP. LRP5/6 is subsequently phosphorylated by CK1γ and GSK3 and will recruit Dvl and Axin. This phosphorylation prevents phosphorylation and subsequent destruction of β-catenin. Stabilized β-catenin translocates to the nucleus where it displaces co-repressor Groucho from TCF, relieving repression and initiating transcription of Wnt target genes. Image modified from Clevers, 2006.

When β-catenin is stabilized during Wnt signaling, it translocates to the nucleus. Here β-catenin interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) (Behrens et al., 1996; van de Wetering et al., 1997), which has 4 homologs in vertebrates. TCF acts as a repressor of Wnt responsive genes by interacting with co-repressor Groucho in the absence of Wnt signals (Cavallo et al., 1998; Roose et al., 1998) (fig 1A). When β-catenin in the nucleus interacts with TCF it replaces Groucho (fig 1B) by binding to a domain of TCF overlapping with the Groucho interaction domain

(Daniels and Weis, 2005). This replacement of Groucho by β-catenin transforms TCF from a repressor into an activator of transcription (van de Wetering et al., 1997) and induces the transcription of Wnt target genes (fig 1B).

There are many different Wnt ligands and receptors, increasing the complexity of Wnt signaling (reviewed by Kikuchi et al., 2009). Different Wnts are able to bind to multiple different Wnt receptors, giving numerous possible ligand-receptor combinations. These different combinations will activate distinct Wnt signaling cascades. There are not only different Fz-Wnt combinations, but different combinations of co-receptors are present as well. Next to LRP5/6, Ror2 and Ryk were shown to be Wnt co-receptors as well. Both Ryk and Ror2 are able to bind to Fz. Ror2 seems to be involved in non-canonical Wnt signaling (Oishi et al., 2003). Ryk was shown to be able to activate the canonical Wnt signaling cascade in mammals (Lu et al., 2004). Giving rise to even more possibilities of Wnt-receptor-co-receptor combinations.

At first it was thought that one Wnt ligand would always give a specific response, but it turned out that a single Wnt ligand can promote different signals, depending on which Wnt receptor it binds. For example Wnt5a was originally thought to act in the non-canonical Wnt pathway, but turned out to be able to activate both pathways, depending on the receptors present. When Ror2 was expressed in the target cells, Wnt5a activated the non-canonical Wnt pathway. However, upon expression of Fz4, the canonical pathway was activated, (Mikels and Nusse, 2006). Thus Wnt signaling is complex and can have different outcomes depending on the cellular context and expression of different signaling components.

In conclusion, the canonical Wnt signaling pathway determines whether its main effector, β-catenin, is stabilized or degraded. Phosphorylation of β-catenin by GSK3 induces the degradation of β-catenin. Upon Wnt signaling this phosphorylation of β-catenin is prevented. The stabilization of β-catenin leads to its translocation into the nucleus and subsequent transcription of target genes. Wnt signaling is a well known regulator of stem cells in both vertebrates and invertebrates such as *C. elegans* and *Drosophila* (reviewed by Eisenmann, 2005; Gonsalves and DasGupta, 2008). How this Wnt signaling influences self-renewal of human and mouse (embryonic) stem cells will be addressed in the next chapter.

Chapter 3 Wnt signaling promotes stem cell self-renewal

Wnt signaling in adult stem cells

Wnt signaling is well known for its role in adult stem cell self-renewal. One of the first indications of the involvement of Wnt signaling in adult stem cells came from the analysis of intestinal crypt stem cells in $Tcf4^{-/-}$ mice. The stem cells of the crypt region in the small intestine of these mice were completely differentiated (Korinek et al., 1998). This indicated that TCF4 is needed for the self-renewal of the crypt stem cells. Dickkopf-1 (Dkk1) is an Wnt antagonist that blocks the canonical Wnt signaling pathway by binding to Wnt receptors. Adenoviral expression of Dkk1 in the crypt stem cell region resulted in loss of proliferation and subsequent loss of crypt stem cells (Kuhnert et al., 2004). This indicates that Wnt signaling is essential for the maintenance of these stem cells. Requirement for Wnt signaling in crypt stem cell maintenance was further supported by in situ hybridization experiments with several Wnt components. These experiments showed that several Wnt ligands and Fz receptors are highly expressed in the crypt stem cell region (Gregorieff et al., 2005). Together these experiments show that Wnt signaling is involved for the maintenance of the crypt stem cells in the small intestine.

Hematopoietic stem (HS) cells also seem to benefit from Wnt signaling. This was shown by prolonged overexpression of β-catenin (Reya et al., 2003). This overexpression can maintain these cells long-term in culture. It inhibits differentiation and promotes self-renewal by controlling transcription of genes involved in the self-renewal of HS cells (Reya et al., 2003). HS cells were also able to activate TCF/LEF-1 reporter constructs *in vivo*, indicating that these cells receive Wnt signals in their normal micro-environment. Inhibition of Wnt signaling in these cells inhibited cell growth, showing that intact Wnt signaling is needed to promote self-renewal (Reya et al., 2003).

These results show that Wnt signals play important roles in the regulation of self-renewal in several adult stem cells. The potency of Wnt signals to promote self-renewal in different tissues suggests that perhaps Wnt signals play a similar role in embryonic stem cell self-renewal.

Wnt signaling in embryonic stem cells

A truncation study of APC was one of the first reports that showed that Wnt signaling might also be involved in the regulation of mES cell pluripotency (Kielman et al., 2002). Different truncation variants of APC were made, missing regions that are important for binding to other proteins of the destruction complex and for binding to β-catenin. When all the β-catenin binding domains were removed, an increase in β-catenin levels was observed. Next to this increase in β-catenin levels, a severe defect in differentiation was observed (Kielman et al., 2002). Similar differentiation defects were observed when a variant of β-catenin was used that is resistant to regulation by the destruction complex. This showed that downregulation of β-catenin is needed for proper differentiation of mES cells (Kielman et al., 2002).

Different expression levels of Wnt receptors affect the outcome of Wnt signaling in ES cells. Transcriptome analysis showed upregulation of Fz5 in hES cells, without detecting expression of any Wnt ligands (Sato et al., 2003). Similar results were found in RT-PCR analysis in mES cells, which revealed that several Frizzleds are upregulated in mES cells, including Fz5 (Hao et al., 2006). Fz5 is known to be able to activate the canonical Wnt signaling pathway together with Wnt5a in *Xenopus* embryos (Ishikawa et al., 2001). Addition of Wnt5a or Wnt3a to mES cells highly expressing Fz5 caused a decrease of phosphorylated β-catenin (Hao et al., 2006), which indicates that canonical Wnt signaling can be activated in these mES cells. One of the genes that was upregulated in these cells is Stat3, which is a component of the LIF pathway and known for its role in mES cell self-renewal. Upregulation of Stat3 by Wnt signals suggests crosstalk between the Wnt and LIF pathways and indicates a potential role for Wnt signaling in mES cell self-renewal. Interestingly, supplying human ES cells with Wnt5a does not seem to inhibit differentiation, in contrast to mouse ES cells receiving the same treatment (Cai et al., 2007). This difference may result from different levels of Fz5, or perhaps

other Fz receptors present in these cells, and emphasizes the importance of the expression of different Wnt receptors on the outcome of Wnt signaling.

A meta-analysis of expression studies provided more evidence for involvement of Wnt signaling in the maintenance of hES cell pluripotency. This analysis used expression data of 38 different studies to create a consensus list of genes highly expressed in hES cells (Assou et al., 2007). One of the genes on this list was Fz7. This Wnt receptor was shown to be downregulated upon differentiation in different studies (Assou et al., 2007). This suggests that Wnt signaling is involved in the maintenance of self-renewal of hES cells and not during differentiation.

The expression studies showed that different Frizzled receptors play a role in Wnt signaling in both human and mouse ES cells. These experiments, together with the truncation experiments of APC and β-catenin, suggest that canonical Wnt signaling is involved in the regulation of self-renewal and differentiation in both human and mouse ES cells.

The use of GSK3 inhibitors further provided evidence for the role of Wnt signaling in maintaining pluripotency of both human and mouse ES cells. When GSK3 inhibitor 6-bromoindirubin-3'-oxime (BIO) was added to mES cells, the pluripotency marker Rex-1 was upregulated (Sato et al., 2004). A dominant negative Tcf3 construct abolished the upregulation of Rex-1, showing that inhibition of GSK3 caused upregulation of this marker. Rex-1 was also upregulated when β-catenin was overexpressed together with Tcf4, confirming that this pluripotency marker is indeed controlled by Wnt signaling. Addition of BIO or recombinant Wnt3a to the hES cells also appeared to maintain the cells in an undifferentiated state with high Oct4 and Nanog expression (Sato et al., 2004). This indicates that both human and mouse ES cells can be maintained in a pluripotent state by addition of GSK3 inhibitor BIO.

More recently it has been reported that a more specific inhibitor of GSK3, CHIR99021 (Bain et al., 2007), could not maintain undifferentiated mES cells on its own, although it did enhance the growth capacity (Ying et al., 2008). However, addition of CHIR99021 together with two inhibitors of the ERK pathway maintained the cells in an undifferentiated state. These cells highly expressed high levels of the pluripotency markers Oct4, Nanog and Rex-1. mES cells treated with both inhibitors showed faster growth rates than cells where LIF and BMP4 was provided and they could be maintained up to 4 weeks (Ying et al., 2008). Knocking out both GSK3α and GSK3β and supplying these cells with ERK inhibitors caused similar results as treatment with CHIR99021 together with the ERK inhibitors (Ying et al., 2008). Others reported that knocking out GSK3α and GSK3β in mES cells severely inhibited differentiation. These double knock out mES cells had elevated levels of TCF-mediated transcription and showed an increase in Oct4 and Nanog expression (Doble et al., 2007). Together these reports provide evidence that GSK3 is involved in the self-renewal of both hES and mES cells.

GSK3 is one of the main players of the canonical Wnt signaling pathway. However, this does not necessarily mean that inhibiting GSK3 is the same as activating the Wnt signaling cascade. Inhibition of GSK3 could also promote other Wnt-independent responses, even if Wnt signaling is shown to be activated by upregulation of Wnt-specific genes. GSK3 functions in multiple pathways beside Wnt signaling, such as the sonic hedgehog (SHH) pathway, and its activation is regulated by multiple pathways as well (reviewed by Jope and Johnson, 2004). Supplying Wnt ligands to ES cells might therefore give a more Wnt-specific effect on the pluripotency and self-renewal of these cells than using GSK3 inhibitors.

The canonical Wnt signaling pathway is be able to inhibit differentiation in mES cells by providing Wnt ligands to these cells. In order to supply mES cells with Wnt signals, conditioned medium from Wnt3a expressing cells is often used. Singla and colleagues reported that Wnt3a conditioned medium could maintain mES self-renewal indefinitely (Singla et al., 2006). Purified Wnt3a, however, was only able to sustain these mES cells for a few days. This shows that Wnt signaling alone is insufficient to

sustain long-term mES self-renewal and that other factors in the conditioned medium might contribute to the self-renewal. Wnt11 conditioned medium could not maintain self-renewal of mES cells, indicating that indeed different Wnt signals can elicit different responses in mES cells (Singla et al., 2006). This difference in response is most likely caused by activation of distinct Wnt signaling pathways; Wnt3a activates the canonical Wnt signaling pathway and Wnt11 the non-canonical Wnt pathway. This supports the idea that canonical rather than the non-canonical pathway is involved in mES self-renewal.

An analysis of several Wnt antagonists showed that addition of Fz8CRD or IWP2 to mES cells reduced self-renewal and induced differentiation. Fz8CRD is a soluble domain of Fz8 that antagonizes Wnt signaling by binding and sequestering Wnt ligands. IWP2 inhibits Wnt signaling by preventing Wnt ligand production. The differentiation of mES cells induced by these antagonist suggests that Wnt signals are produced and secreted by the mES cells to prevent differentiation. Indeed, addition of Wnt3a rescued self-renewal of the Fz8CRD and IWP2 treated mES cells, showing that Wnt signaling is needed for self-renewal in mES cells (ten Berge et al., 2011). Addition of Wnt3a together with LIF was the minimal requirement of growth factors needed to maintain mES self-renewal. Withdrawal of LIF caused loss of the cells, indicating that Wnt signaling on its own is insufficient to sustain self-renewal (ten Berge et al., 2011). Supplying mES cells with Wnt3a also inhibited differentiation in differentiation promoting medium (ten Berge et al., 2011). These observations confirm an older report that providing mES cells with Wnt antagonist SFRP2 promotes neural differentiation, which was blocked by forced expression of Wnt1 (Aubert et al., 2002). Together these results show that activation of canonical Wnt signaling by supplying mES cells with Wnt ligands is sufficient to inhibit differentiation but, although needed for self-renewal, canonical Wnt signaling is insufficient to promote self-renewal on its own (ten Berge et al., 2011).

Slightly different results were obtained when the previously described GSK3 inhibitor CHIR99021 was used. CHIR99021 was not as effective as Wnt3a in combination with LIF in supporting self-renewal. Interestingly, CHIR99021 combined with ERK inhibitors supported self-renewal, similar to ERK inhibitors combined with Wnt3a (ten Berge et al., 2011). This shows that blocking ERK signaling together with activation of Wnt signaling is sufficient to maintain self-renewal. However CHIR99021 was able to do this to a greater extent than Wnt3a in combination with ERK inhibitors (Ying et al., 2008). This indicates that activation of Wnt signaling by the same treatment sometimes can give different responses when performed by different groups. On the other hand, both show that Wnt signaling is able to support self-renewal at least in part by inhibiting differentiation, despite the differences. This provides more evidence for a role of Wnt signaling in maintaining mES cell self-renewal.

Taken together these results show that activation of canonical Wnt signaling, by supplying ES cells with either Wnt ligands or GSK3 inhibitors, prevents differentiation. Besides preventing differentiation, canonical Wnt signaling is clearly involved in maintaining self-renewal, although canonical Wnt signaling is insufficient to do this on its own. Wnt signaling not only plays a role in the self-renewal of ES cells, it is also involved during the reprogramming process and in the maintenance of self-renewal in iPS cells.

Wnt signaling in induced pluripotent stem cells

Several reports have shown that Wnt signaling is important during the reprogramming and maintenance of iPS cells. In human iPS cells it was shown that providing small molecule Wnt inhibitors facilitated BMP4 induced cardiac differentiation (Ren et al., 2011). This indicates that Wnt signals blocked differentiation of iPS cells, similar to ES cells. Multiple reports have shown that Wnt signaling can also facilitate the reprogramming process. c-Myc was among the first factors used to generate iPS cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). However, the use of c-Myc increases tumorigenesis (Okita et al., 2007), thus preferentially this factor is not used for the induction of pluripotency. iPS cells have been obtained without c-Myc, but this greatly reduces the

efficiency of the process (Wernig et al., 2008). This resulted in the search for factors that could replace c-Myc in the reprogramming process. When transfected fibroblasts were grown on Wnt3a cultured medium the reprogramming of these cells was enhanced in the absence of c-Myc (Marson et al., 2008a). The presence of Wnt3a during the reprogramming most likely aids the process at least in part by activating c-Myc transcription. The same result could not be obtained when using GSK3 inhibitors, quite possibly because the half-life of these inhibitors is too low (Marson et al., 2008a). However, in a later study CHIR99021 was shown to be able to facilitate reprogramming of mouse embryonic fibroblasts (MEFs) with only 2 factors, Oct4 and Klf4. CHIR99021 effectively replaced Sox2 in the reprogramming process (Li et al., 2009). During the reprogramming of human cells the same results were found. When neural stem cells where used to generate iPS cells, GSK3 and ERK inhibitors were needed to obtain mouse iPS cells with stable expression of pluripotency genes Oct4 and Nanog (Silva et al., 2008).Together these result show that reprogramming of both mice and human fibroblasts can benefit from Wnt signaling.

Besides reprogramming with the use of viral vectors, cell fusion mediated reprogramming benefits from Wnt signaling as well. Cell fusion is an old technique used to reprogram differentiated cells. When this technique was first developed, somatic cells were fused with embryonic carcinoma (EC) cells and were reprogrammed to a pluripotent state (Miller and Ruddle, 1976). ES cells can reprogram somatic cells just like EC cells (Tada et al., 2001). It turned out that supplying the cells during the fusion process with Wnt3a greatly enhanced the amount of reprogrammed colonies formed. The beneficial effect seemed to be reduced after treatment for longer than 24 hours (Lluis et al., 2008). Similar results were found when BIO was used instead of Wnt3a. This showed that at least short-term activation of Wnt signaling can assist in the reprogramming of these cells. Pre-treating the mES cells before the fusion with Wnt3a or BIO turned out to enhance the reprogramming.
When the cells were treated for either 24 or 96 hours prior to cell fusion, this resulted in an increase in reprogramming. However, treatment for 12, 38 or 72 hours did not result in a higher reprogramming efficiency. This indicated that a periodic activation of the Wnt signaling is able to support the reprogramming process (Lluis et al., 2008). β-catenin protein levels turned out to be the highest at these time points, indicating periodic accumulation of β-catenin is needed to enhance the reprogramming ability of these cells. Thus, canonical Wnt signaling is able to enhance both cell fusion mediated reprogramming and induced reprogramming with the use of viral vectors, in both human and mouse cells.

Taken together, these reports show that not only adult stem cells, but also embryonic- and induced pluripotent stem cells can benefit from Wnt signaling. In embryonic- and induced pluripotent stem cells it has a beneficial effect on both self-renewal and pluripotency. However, from these reports it is not clear how Wnt signaling is able to promote the self-renewal of ES cells. It is known that activation of Stat3 and c-Myc by Wnt signaling could contribute to the self-renewal of ES cells, but an increase of core pluripotency genes, Oct4 and Nanog has been observed as well. Thus, Wnt signaling might also play a role in the core transcriptional regulatory circuitry. The next chapter will describe how Wnt signaling is able to affect the core regulatory gene network of pluripotency.

Chapter 4 Wnt signaling acts on the core transcriptional regulators

Several reports showed how Wnt signaling is able to promote self-renewal and pluripotency in ES cells. Although different groups implicate different mechanisms, most find that canonical Wnt signaling acts on the core regulatory transcriptional gene network of pluripotency. Wnt signaling could promote pluripotency by relieving the TCF3 mediated inhibition of core pluripotency regulators, by direct interaction of β-catenin with components of the core regulatory circuitry or by TCF1/LEF1 mediated gene activation. This chapter discusses the current hypotheses of how Wnt signaling is able to promote pluripotency and self-renewal in ES cells.

TCF3 is an integral component of the core transcription circuitry

A genome wide binding profile for TCF3 indicated how Wnt signaling might affect the core transcriptional regulatory circuitry of pluripotency. Three earlier findings suggested that TCF3 might be involved in the regulation of mES cell pluripotency: Wnt signaling is involved in the regulation of pluripotency of mES cells and TCF3 is a main player of Wnt signaling, TCF3 is highly expressed in mES cells, and $Tcf3^{-/-}$ mES cells showed increased Nanog expression (Pereira et al., 2006). The binding profile showed that TCF3 occupies more than 1000 promoters, including known Wnt regulated genes, and has an overlapping target gene profile with Oct4, Sox2 and Nanog (Boyer et al., 2005; Cole et al., 2008; Loh et al., 2006). Another independent genome wide binding profile confirmed the overlapping target genes with Oct4 and Nanog (Tam et al., 2008). This suggests that TCF3 might affect the regulation of Oct4, Sox2 and Nanog of these genes. Oct4, Nanog and TCF3 also showed the same spatial distribution pattern from the transcription start sites among the promoters occupied by these transcription factors (Cole et al., 2008). This shows that Oct4, Nanog and TCF3 bind at the same position on the promoters. Oct4 and Sox2 are known to form heterodimers, therefore these promoters are most likely occupied by all four transcription factors. The promoters of Oct4, Sox2, Nanog and TCF3 were among the promoters bound by these four factors, showing that TCF3 is an integral component of the auto-regulatory gene network regulating pluripotency (Fig 2). Wnt3a is not able to induce self-renewal in the absence of Nanog, suggesting that Wnt signaling indeed works through the core transcriptional regulatory gene network (Yi et al., 2011). An explanation for the role of TCF3 in the core regulatory transcriptional circuitry would be that TCF3 acts as a repressor on this regulatory gene network. Upon activation of Wnt signaling this repression would be relieved by binding of β-catenin to TCF3.

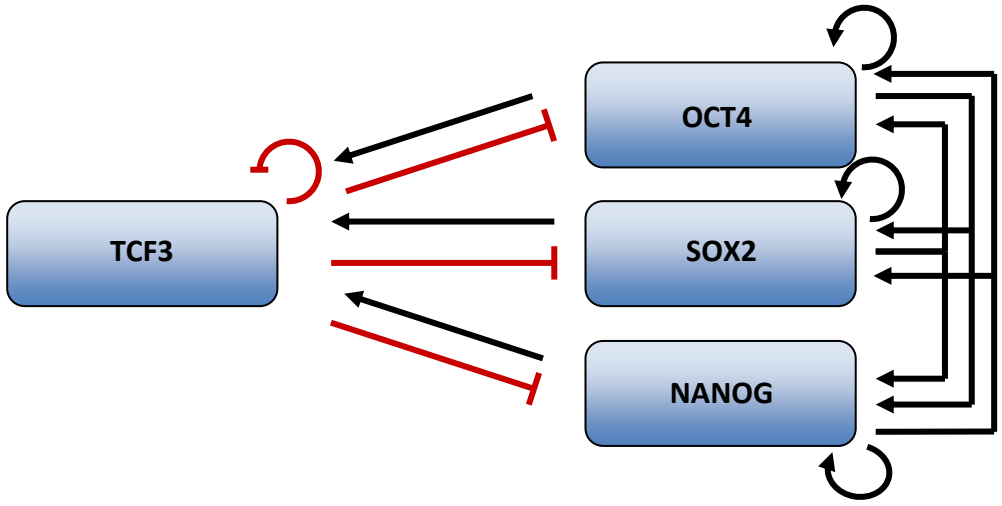

Figure 2 Interplay of Tcf3 with the core regulatory gene network of pluripotency. Oct4, Sox2 and Nanog all promote each other's and their own transcription as well as the transcription of Tcf3. Tcf3 inhibits the transcription of Oct4, Sox2, Nanog and itself. Black arrows indicate promotion of transcription, red arrows indicate repression.

Several Tcf3 deletion experiments confirmed the suggestion that the primary role of TCF3 is to repress Oct4, Sox2 and Nanog expression and thereby to inhibit self-renewal. Two independent Tcf3 knock down experiments using RNA interference (RNAi) showed upregulation of several genes bound by TCF3. Oct4, Sox2 and Nanog were among the upregulated genes, indicating that TCF3 represses these genes in the absence of Wnt signaling (Cole et al., 2008; Tam et al., 2008). Similar results were obtained after addition of Wnt3a conditioned medium, suggesting that activation of Wnt signaling activates components of the core transcriptional network by de-repression of TCF3 (Cole et al., 2008). Earlier results also suggested a repressive role of TCF3 on the pluripotency genes. Knock out of Tcf3 in mES cells resulted in delayed differentiation. This delay was caused at least in part by elevated levels of Nanog expression (Pereira et al., 2006).

If TCF3 represses the pluripotency genes, it is possible that TCF3 is needed for repression of these genes during differentiation and thus needed for proper differentiation. Indeed, RNAi knock down of Tcf3 inhibited retinoic acid (RA) mediated differentiation of mES cells. Tcf3 knock down allowed mES to self-renew and retain their pluripotency for several days in media containing RA (Tam et al., 2008). These cells maintained self-renewal upon LIF withdrawal (Tam et al., 2008; Yi et al., 2008), resembling the results obtained after Wnt3a supplementation (ten Berge et al., 2011). This suggests that Wnt signaling works at least in part by inhibiting repression of TCF3 on the pluripotency genes. $Tcf3^{-/-}$ mES cells could be maintained in the absence of LIF for at least 30 days (Yi et al., 2008). Combining Tcf3 knock out with ERK inhibitors showed an increase in self-renewal of mES cells, resembling results obtained with ERK inhibitors and GSK3 inhibitors (Yi et al., 2011). However, addition of GSK3 inhibitors to $Tcf3^{-/-}$ mES cells only slightly increases the self-renewal capacity of these cells, suggesting that GSK3 inhibition mainly works through TCF3 (Yi et al., 2011). Together these reports indicate that Wnt signaling is able to suppress differentiation of mES cells by relieving the repression of TCF3 on the pluripotency genes, through inhibition of GSK3 mediated phosphorylation of β-catenin.

The repressive role of TCF3 on the core pluripotency genes was confirmed by overexpression experiments with Tcf3. Overexpression of Tcf3 induced differentiation in mES cells, while the differentiation was strongly inhibited by supplying the cells with Wnt3a (Yi et al., 2011). This further suggests a repressive role of TCF3 on self-renewal, probably by repressing the genes of the core transcriptional regulatory circuitry. More evidence for an integral role of TCF3 in the regulatory circuitry of pluripotency was found by RNAi knock down of Oct4 and Nanog. This showed that Tcf3 expression decreased after Oct4 RNAi and slightly decreased after Nanog RNAi, indicating that Tcf3 is itself regulated by the core transcriptional network (Yi et al., 2008).

The previous results showed that TCF3 represses the core transcriptional regulatory network but did not show whether these effects are direct. Direct effects could result from either TCF3 binding to these transcription factors or TCF3 displacing them from the promoters. A Chromatin immunoprecipitation followed by sequencing (ChIP-seq) experiment showed more precise binding locations of OCT4, SOX2, NANOG and TCF3 (Fig 3)(Marson et al., 2008b). These results confirmed earlier observations that OCT4, SOX2, NANOG and TCF3 bind similar regions in the genome (Tam et al., 2008), as exemplified in figure 3A for the region surrounding the Sox2 gene. When looking at these binding locations in more detail it becomes clear that OCT4, SOX2 and NANOG show an almost identical binding distribution (Fig 3B)(Marson et al., 2008b). Despite showing a slightly different pattern, TCF3 binding is very similar to OCT4, SOX2 and NANOG and all four transcription factors cluster around OCT4/SOX2 binding motifs (Fig 3B). This suggest that TCF3 directly represses OCT4, SOX2 and NANOG activity at promoters occupied by all four factors. Whether this repression happens by binding to these transcription factors, or by displacing them from the promoters remains to be elucidated.

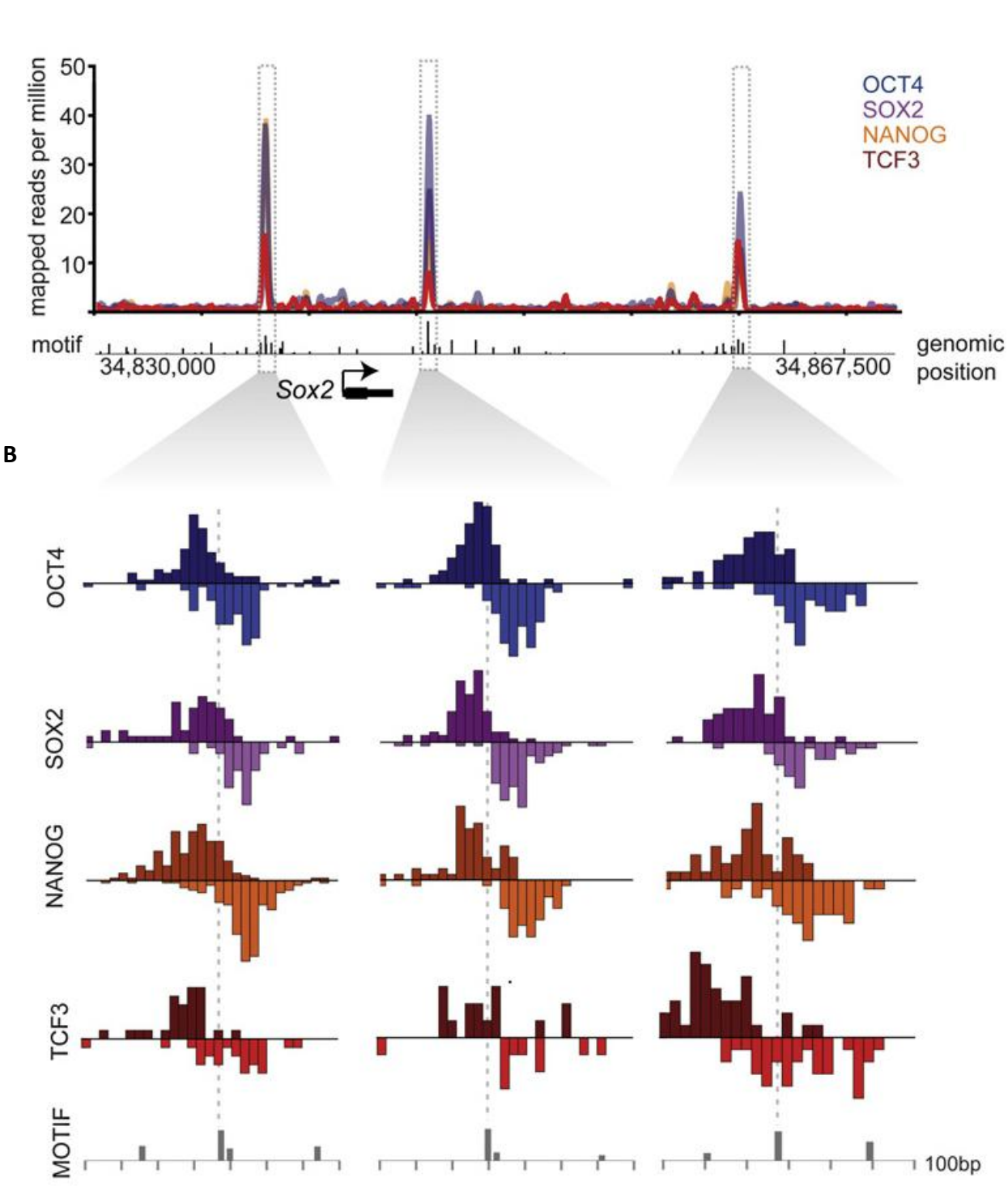

Figure 3 Binding of OCT4, SOX2, NANOG and TCF3 at the genomic region surrounding the Sox2 gene. A ChIP-seq reads of OCT4 (blue), SOX2 (purple), NANOG (orange) and TCF3 (red) in the mouse genomic region around the SOX2 gene. **B** Zoom-in of the three regions with binding of the four transcription factors. The 5' base pair reads were separated by sense (dark coloured) and anti-sense (light coloured) strand and binned in 25 bp regions. The reads are directed to the OCT4/SOX2 binding motif indicated in the bottom. Modified from Marson et al., 2008b.

Together, results from the Tcf3$^{-/-}$ knock out experiments, the RNAi knockdown of Tcf3, Oct4 and Nanog and the GSK3 inhibitor experiments confirm that Oct4, Sox2, Nanog and TCF3 regulate each other's transcription (Fig. 2). This leads to the following model; when Wnt signaling is present, GSK3 activity is inhibited. The inhibition of GSK3 promotes β-catenin mediated de-repression of TCF3 targets such as Nanog, Oct4 and Sox2. These proteins will in turn bind to- and activate genes important for self-renewal and pluripotency, including their own promoters. In addition, other effects of Wnt signaling, such as stabilization of c-Myc may contribute to the maintenance of pluripotency.

TCF3 independent effects on self-renewal

Not only TCF3 is involved in the maintenance of pluripotency in ES cells, TCF1 and LEF1 also appear to play a role in this process, by activating the components of the core regulatory circuitry of pluripotency. Tcf3 is the highest expressed TCF in mES cells, but other TCFs are present as well (Anton et al., 2007; Pereira et al., 2006; Yi et al., 2011). Co-expression of a stabilized form of β-catenin with LEF1 in embryonic kidney (EK) cells greatly induced an Oct4-promoter-reporter construct (Li et al., 2012), showing that LEF1 can regulate β-catenin mediated transcription of Oct4. Endogenous TCF1 and LEF1 activate a Tcf-reporter construct upon addition of stabilized β-catenin in mES cells, where TCF1 was the most potent of the two (Yi et al., 2011). This shows that TCF1 and LEF1 activate β-catenin dependent transcription in mES cells. Knock down of Tcf1 in Tcf3$^{-/-}$ mES cells resulted in reduced growth, indicating that both TCF1 and TCF3 contribute to the self-renewal of mES cells (Yi et al., 2011). When Tcf1 was knocked down using RNAi in Tcf3 expressing mES cells, a reduction was found in expression levels of several pluripotency genes. This further shows that TCF1 might play a role in maintaining self-renewal. A TCF3 variant that is unable to interact with β-catenin showed inhibition of β-catenin dependent TCF3 de-repression. This TCF3 variant also inhibited β-catenin dependent TCF1 activation, although TCF1 action was not fully blocked (Yi et al., 2011) . This suggests two distinct roles for TCF3 and TCF1 in the maintenance of self-renewal. TCF1 activates transcription upon activation of Wnt signaling and TCF3 represses, at least in part, this transcription.

The above mentioned results suggest that repression of β-catenin by GSK3 works on self-renewal through TCF3, TCF1 and LEF1. However, there might be β-catenin independent effects of GSK3 inhibition. Analysis of β-catenin$^{-/-}$ mES cells showed that these cells had lower expression of several pluripotency markers, although Oct4, Sox2 and Nanog seemed largely unaffected (Anton et al., 2007). Others found that β-catenin$^{-/-}$ mES cells could grow much faster in medium containing ERK inhibitors, LIF and CHIR99021 (Wray et al., 2011). Surprisingly, these cells had a reduced self-renewal capacity in medium with only ERK inhibitors and LIF (Wray et al., 2011), suggesting that there are still β-catenin independent effects of inhibiting GSK3 that promote self-renewal, perhaps through regulation of c-Myc stability (Gregory et al., 2003). This indicates that although β-catenin is involved, it is not strictly necessary for self-renewal.

A truncated version of β-catenin, missing its C-terminal transactivational domain was unable to activate TCF-reporters. However, when this truncated version of β-catenin was expressed in β-catenin$^{-/-}$ cells, differentiation could still be inhibited by CHIR99021 (Wray et al., 2011). This indicates that β-catenin has an activation domain independent function in maintaining self-renewal. One of these activation domain independent functions could be inhibition of TCF3, as this variant of β-catenin is still able to bind to TCF3. The truncated β-catenin variant could still relieve repression of genes targeted by TCF3. Besides its role in activation of TCF-mediated transcription and its role in TCF3 inhibition, a third role for β-catenin has been proposed where β-catenin directly interacts with Oct4.

Direct interaction of β-catenin with Oct4

The previous paragraphs have shown that TCFs have prominent roles in the maintenance of ES cell self-renewal. In addition, a direct interaction of Oct4 with β-catenin has been proposed to play a role in the regulation of ES cell self-renewal as well. Takao and colleagues found that expression of a stabilized version of β-catenin could maintain mES cells in culture in the absence of LIF (Takao et al., 2007). These cells could be cultured for up to one month, showed inhibited differentiation and had an upregulation of pluripotency markers such as Oct4 and Nanog (Takao et al., 2007). To investigate how β-catenin was able to activate Nanog expression, they deleted the Oct4 binding sites in the Nanog promoter region. β-catenin was no longer able to regulate Nanog expression when the Oct4 binding sites were mutated in the Nanog promoter, showing that β-catenin regulates Nanog transcription in an Oct4 dependent manner in mES cells. This suggests that β-catenin somehow works together with Oct4 to activate Nanog expression. A GST pull-down assay showed that β-catenin is able to directly bind to Oct4. Together these results made the authors suggest that Oct4 and β-catenin form a complex together to activate Nanog expression (Takao et al., 2007).

A few years later similar results were found; overexpression of β-catenin in mES cells inhibited differentiation and phenocopied knockout of GSK3 in mES cells. Both GSK3 knockout and β-catenin overexpression caused high expression of Oct4 and Nanog (Kelly et al., 2011). Knockdown of β-catenin by RNAi rescued the differentiation defects in the GSK3 double knockout mES cells, which confirms that the effects of GSK3 depletion were mediated by β-catenin. This suggests that GSK3 at least might work through β-catenin to activate Oct4 expression. The same authors used co-immunoprecipiation to show that β-catenin directly interacts with Oct4 (Kelly et al., 2011). To investigate the effect of β-catenin on Oct4 mediated transcription they used an Oct4 binding domain-reporter construct. Wnt3a and CHIR99021 were able to activate this reporter. A β-catenin variant missing its transactivation domain similarly activated this reporter. This suggests a transactivation independent Oct4 activation by β-catenin (Kelly et al., 2011), perhaps through inhibition of TCF3.

It is still a topic of debate whether Oct4 and β-catenin directly bind to each other, or whether this interaction is mediated by other proteins. As shown previously, β-catenin is known to interact with TCF3, which in turn co-occupies many promoters together with Oct4. Thus it is possible that the observed β-catenin-Oct4 interaction is mediated by TCF3. The use of a transactivational deficient β-catenin showed that the transactivational activity of β-catenin is not necessary for Oct4 activation. However, this does not exclude the possibility that β-catenin mediates this activation by de-repression of TCF3, since this truncated version of β-catenin is still able to bind to TCF3. Yi and colleagues presented some evidence against the model of direct β-catenin-Oct4 binding by analyzing CHiP-qRT-PCR data in Tcf3-/- mutants. If Oct4 binds β-catenin, it should recruit β-catenin to the chromatin in $Tcf3^{-/-}$ cells. However, in $Tcf3^{-/-}$ mES cells, β-catenin occupancy was reduced (Yi et al., 2011). This suggests that Tcf3 rather than Oct4 recruits β-catenin to the chromatin. However, there were still low levels of β-catenin present at the chromatin in $Tcf3^{-/-}$ Tcf1 RNAi cells, showing that perhaps a low level of β-catenin is bound by Oct4. If TCF3 would mediate the observed interaction between β-catenin and Oct4, this would not explain how β-catenin was able to bind to Oct4 in a GST-pull down assay using bacterial lysates (Takao et al., 2007). This shows that Oct4 likely is able to bind β-catenin, but does not necessarily mean that they promote transcription together. Taken together it is still unclear what the precise role of the Oct4-β-catenin interaction is during mES cell self-renewal.

In conclusion, Wnt signaling is able to promote self-renewal, to inhibit differentiation and to upregulate several transcription factors known for their role in pluripotency, including Oct4 and Nanog. The co-occupancy of TCF3 with components of the core transcriptional regulatory circuitry suggests that TCF3 is an integral component of this regulatory gene network (Fig 2). Tcf3 deletion and overexpression experiments showed that TCF3 works mainly as a repressor of these genes. How TCF3 exactly represses this network, either by displacing OCT4, SOX2 and NANOG from the target genes or by binding to these transcription factors is so far unknown. This repression by TCF3 provides

a model for how GSK3 inhibitors or Wnt signals can promote self-renewal in ES cells: activation of Wnt signaling or small molecule GSK3 inhibitors would prevent the phosphorylation of β-catenin and thereby prevent its degradation. β-catenin will subsequently bind to TCF3, which relieves the TCF3 repression on the core regulatory gene network of pluripotency. These genes would then activate the rest of the network mediating pluripotency and thereby promote self-renewal and pluripotency. Even though most of the evidence shows a prominent role of TCF3 de-repression by β-catenin in maintaining ES cell self-renewal, TCF1 and LEF1 mediated gene expression and a direct interaction of β-catenin with Oct4 might also play a role in maintaining mES cell pluripotency and self-renewal.

Most of the reports so far have primarily shown a beneficial effect of Wnt signaling on mES cell self-renewal and have shown that Wnt signaling inhibits differentiation. However, there are also several contrasting reports, showing that Wnt signaling promotes differentiation. The next chapter will discuss these reports.

Chapter 5 Wnt signaling promotes embryonic stem cell differentiation

In the previous two chapters many reports were discussed that show how Wnt signaling is important for ES cell self-renewal. This chapter will discuss the conflicting reports about Wnt signaling promoting differentiation, and will propose possible explanations for the differences of the effect of Wnt signaling on ES cells.

One of the earliest suggestions that Wnt signaling might promote differentiation came from an analysis of the expression levels of several Frizzleds and Wnt antagonists upon RA induced differentiation in hES cells. Several Frizzleds were upregulated and several Wnt antagonist were downregulated upon differentiation (Walsh and Andrews, 2003), suggesting that Wnt signaling plays a positive role during this process. However, also some Frizzleds were downregulated and several Wnt antagonists were upregulated upon differentiation, which suggests a role for Wnt signaling in self-renewal and pluripotency. Moreover, as mentioned earlier, not all Wnt receptors will activate the same Wnt signaling pathways. For example, Fz6, which has been shown to repress the canonical Wnt signaling pathway in human EK cells (Golan et al., 2004), was upregulated during differentiation, indicating that the canonical Wnt signaling pathway is antagonized. On the other hand, Fz1 was upregulated upon RA addition to the hES cells (Walsh and Andrews, 2003). Fz1 was shown to induce the canonical Wnt signaling pathway in EK cells (Golan et al., 2004), which shows that canonical Wnt signaling coincides with differentiation. Thus it is hard to conclude from only receptor expression studies which of the Wnt pathways is activated upon differentiation and whether it is needed for self-renewal or for differentiation. It is quite possible that canonical Wnt signaling might be important in both processes and other signaling pathways might determine whether the cell remains pluripotent or starts to differentiate.

Not only expression studies suggested a role for Wnt signaling in differentiation, but experiments where ES cells were supplied with Wnt signals as well. Providing mES cells with Wnt3a conditioned medium resulted in neural differentiation of these cells (Otero et al., 2004). Overexpression of β-catenin gave similar results. Both the results of the Wnt3a conditioned medium and the overexpression of β-catenin are in sharp contrast with other studies in which Wnt3a conditioned medium did not induce differentiation but was reported to promote self-renewal of ES cells (Sato et al., 2004; Singla et al., 2006; ten Berge *et al.,* 2011). Other studies showed that overexpression of stabilized β-catenin led to an increase in self-renewal and impaired neural differentiation after 14 days of embryonic body formation (Kelly et al., 2011). The observed differences might come from differences in the cell lines used, or, in the case of the Wnt3a conditioned medium, from differences in the cells that were used to create the conditioned medium. If the two conditioned media contained different signaling molecules, these signaling components might have influenced the outcome of Wnt signaling. Another possibility for the difference in outcome of β-catenin overexpression might come from the state of the cells used. When Otero and colleagues analyzed the mES cells they had already formed embryonic bodies, while Kelly and colleagues analyzed the cells before that stage. These different cell states might have influenced the effect of Wnt signaling on ES cell pluripotency.

Interestingly, not all reports that show differentiation upon induction of Wnt signaling show commitment into the same lineage. The above mentioned report suggested that Wnt signaling is important for neural differentiation, whereas others reported that Wnt signaling is required for mesoderm/endoderm specific differentiation in both human and mouse ES cells (Bakre et al., 2007; Lindsley et al., 2006). Activation of Wnt signaling either by supplying cells with Wnt3a or by inhibiting GSK3 resulted in the upregulation of certain mesoderm and endoderm specific markers in human and mouse ES cells. These cells were named mesendodermal progenitor cells (Bakre et al., 2007). The progenitor cells could still be maintained long term in culture despite the partial differentiation, suggesting that Wnt signaling might be responsible for self-renewal of these cells. When mES cells

were induced to differentiate and simultaneously supplied with Wnt antagonist Dkk1, this resulted in downregulation of mesodermal and endodermal markers, showing that Wnt signaling is needed during differentiation into these lineages (Lindsley et al., 2006). Davidson and colleagues found that activation of Wnt signaling either by supplying hES cells with Wnt3a or by inhibiting GSK3 reduced self-renewal (Davidson et al., 2012) and that a β-catenin reporter construct was inactive in undifferentiated hES cells. This suggests that active Wnt signaling is not needed for self-renewal of these cells. They conclude from these results that Wnt mediated β-catenin activation is associated with differentiation rather than self-renewal. However, an inactive β-catenin reporter construct does not necessarily indicate that Wnt signaling does not play a role in undifferentiated hES cells. For example β-catenin induced de-repression of TCF3 might still be important for self-renewal of these cells.

Together, these reports suggest a prominent role for Wnt signaling during differentiation. However, the requirement of Wnt signaling during differentiation does not exclude the possibility that Wnt signaling is important for ES cell self-renewal. Wnt signaling most likely is involved in both processes. The involvement of Wnt signaling in both processes suggests that Wnt signaling is important for maintaining self-renewal of ES cells, but once the cells start to differentiate active Wnt signaling is needed to guide the cells in the right direction.

A different role for the Oct4-β-catenin interaction in hES cells was proposed as well. The interaction of β-catenin with Oct4 was found in mES cells (Kelly et al., 2011; Takao et al., 2007), and also in human ES and EK cells (Abu-Remaileh et al., 2010). However, in this last report the interaction was found to be important for Oct4 mediated degradation of β-catenin. This study showed that β-catenin was upregulated and Oct4 was downregulated upon RA induced differentiation in hES cells, suggesting involvement of Wnt signaling in differentiation (Abu-Remaileh et al., 2010). Removal of Oct4 in hES cells resulted in an increase of β-catenin protein levels, and activation of Oct4 in hEK cells resulted in a decrease of β-catenin protein levels (Abu-Remaileh et al., 2010). This suggests that Oct4 induces degradation of β-catenin. Protease inhibitors and mutations in APC inhibited Oct4 induced β-catenin degradation, indicating that Oct4 works through APC to induce the degradation of β-catenin. Oct4 is not only able to bind to β-catenin, but to Axin as well, suggesting that Oct4 has a direct role in the regulation of β-catenin stability (Abu-Remaileh et al., 2010). Oct4 is well known for its role in undifferentiated ES cells, thus this Oct4 mediated degradation of β-catenin suggests that β-catenin is not important for the undifferentiated state but is associated with differentiation.

In line with the findings of Abu-Remaileh and colleagues, others reported that Oct4 activity is able to suppress β-catenin mediated transcription (Davidson et al., 2012). However, it is possible that the downregulation of β-catenin is part of a negative feedback loop involving Oct4, TCF3 and β-catenin. At least in mES cells, TCF3 represses Oct4 expression, which is de-repressed by β-catenin and thereby elevates Oct4 levels. When OCT4 levels are sufficiently high, OCT4 mediates the degradation of β-catenin. Degradation of β-catenin leads to repression of Oct4 by TCF3, preventing OCT4 levels from becoming too high. OCT4 levels are known to be tightly regulated as too high or too low expression induces differentiation (Niwa et al., 2000). The regulation of β-catenin levels by Oct4 might represent a way to stabilize Oct4 levels rather than a way to prevent Wnt induced differentiation.

Differences in cell lines or duration of the induction might contribute to the conflicting results found in the effects of Wnt signaling on ES cells. Whereas the downregulation of β-catenin mediated transcription in ES cells can be explained as a negative feedback on Oct4 transcription, the opposing effects of Wnt induction on self-renewal between the studies described in this chapter and the third chapter is harder to explain. One of the main factors involved is duration of Wnt induction. Davidson and colleagues activated Wnt signaling for one or two weeks (Davidson et al., 2012), while others only induced for several days (Cai et al., 2007; Singla et al., 2006; ten Berge et al., 2011). Perhaps Wnt

signaling is only able to promote short term and not long term self-renewal in ES cells, although Wnt mediated long term self-renewal has been reported in mES cells (Miyabayashi et al., 2007; Singla et al., 2006; Ying et al., 2008). Differences in cells lines used may also contribute to the differences in the effects of Wnt signaling on ES cells, as proposed by Davidson and colleagues. Perhaps the cell lines they used were more primed for differentiation as the ones used by others (Davidson et al., 2012). Most of the reports showing that Wnt signaling is important in ES cell self-renewal used mES cells, especially the reports that showed the effects of TCF3 on the core regulatory gene network, whereas most of the reports showing differentiation upon Wnt signaling used hES cells. Thus, perhaps the differences of the effects of Wnt signaling on ES cells are in part caused by species specific differences.

CBP and p300, switch between proliferation and differentiation

There might also be another mechanism involved in the outcome of Wnt signaling. Two histone acetyltransferases, CBP and p300, may determine whether Wnt signaling induces proliferation or differentiation. CBP and p300 have been shown to be co-activators of β-catenin dependent transcription (Hecht et al., 2000) and they have divergent functions in Wnt signaling. In cells derived from the neural crest lineage, β-catenin promotes self-renewal together with CBP. In contrast, the β-catenin/p300 interaction promotes differentiation (Teo et al., 2005). Switching between these co-factors of β-catenin could determine the outcome of Wnt signaling. CBP as co-activator promotes self-renewal and pluripotency, while p300 as co-activator induces differentiation

One explanation of this differential activity of CBP and p300 as co-activator could be a difference in recruited co-factors by CBP and p300. Ma and colleagues showed that upon binding of p300/β-catenin to the survivin promoter SUMO-1 is recruited (Ma et al., 2005). SUMO-1 is known to modify p300, which subsequently recruits histone deacetylase HDAC6 and represses p300 bound target genes (Girdwood et al., 2003). When bound to the survivin promoter CBP/β-catenin together activate survivin expression while p300/β-catenin recruit HDAC6 and represses survivin expression. Similar modes of regulation might occur at other Wnt target genes important for the regulation of pluripotency and differentiation.

In a chemical library screen a compound, IQ-1, was found that was able to maintain mES cells long term in the absence of LIF in a Wnt dependent manner (Miyabayashi et al., 2007). IQ-1 turned out to bind to PR72/PR130, a subunit of the protein phosphatase PP2A. PR72/130 are known to bind to naked cuticle (Nkd) and together with Nkd able to modulate Wnt signaling (Creyghton et al., 2005). Miyabayashi and colleagues hypothesized that IQ-1 might influence the switch between CBP and p300 as co-activator for β-catenin mediated transcription. Indeed treating the ES cells with Wnt3a and IQ-1 resulted in an increase of CBP bound to β-catenin at the expense of p300 bound by β-catenin (Miyabayashi et al., 2007). Phosphorylation of p300 is necessary for p300-β-catenin interaction. Addition of IQ-1 removes this phosphorylation of p300, reducing the interaction with β-catenin (Miyabayashi et al., 2007). IQ-1 breaks the interaction of the PP2A/PR73/PR130/Nkd complex, freeing the phosphatase activity of PP2A to remove the phosphorylation on p300. From these results the authors concluded that the switch between p300 and CBP as co-factor for β-catenin is mediated by the PP2A complex (Fig 4) (Miyabayashi et al., 2007). In support of this model they found that Wnt3a alone was unable to support long-term mES cell self-renewal. However, in combination with IQ-1 the mES cells could be maintained up to 48 days (Miyabayashi et al., 2007).

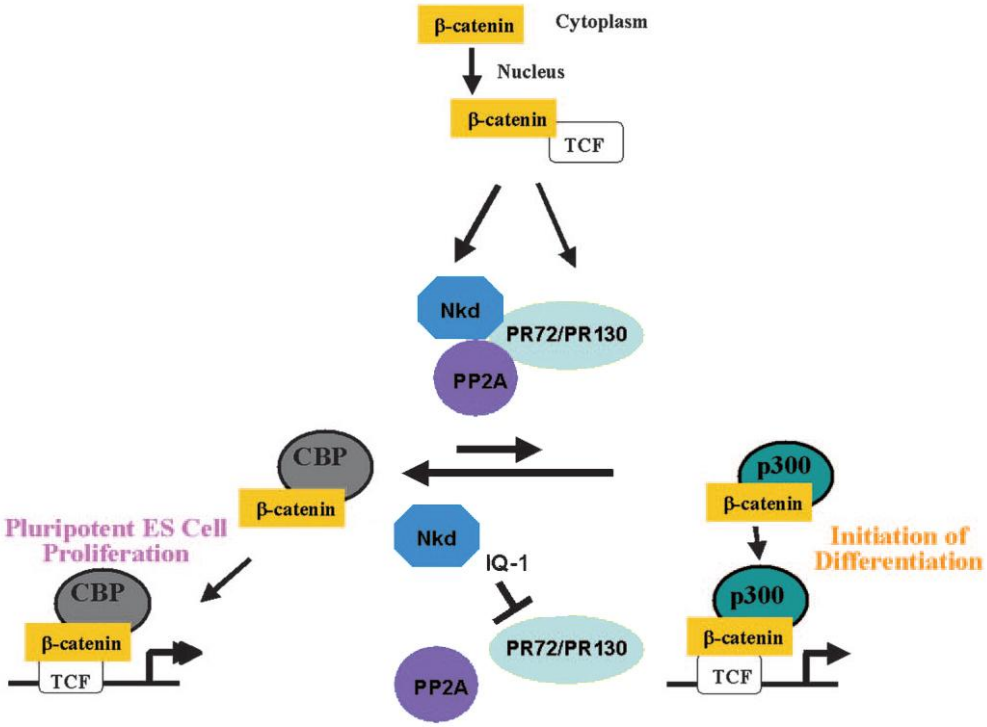

Figure 4 Model of how the Nkd/PP2A/PR72/PR130 complex influences the outcome of Wnt signaling. Upon activation of Wnt signaling β-catenin translocates to the nucleus where it interacts with TCFs. CBP and p300 both can act as a co-activator, which is controlled by the levels of phosphorylated p300. IQ-1 breaks the Nkd/PP2A/PR72/PR130 complex. This frees the phosphatase activity of PP2A, which leads to a decrease in p300 phosphorylation and thereby promotes the interaction of CBP with β-catenin. Image modified from Miyabayashi et al., 2007.

This model could perhaps explain why the results of the effect of Wnt signaling on ES cells contradict each other. If the complex of Nkd/PP2A/PR73/PR130 determines the outcome of Wnt signaling, perhaps this complex is differentially regulated in these different experiments. p300 would primarily be a co-activator in ES cells that start differentiation and CBP might be a co-activator in cells that remain pluripotent and continue to self-renew. Although this raises the question; if this phosphatase complex determines the outcome of Wnt signaling, how is this complex regulated? It is possible that other signaling pathways influence the stability of the PP2A/PR73/PR130/Nkd complex, thereby regulate PP2A activity and thus influence the effect of Wnt signaling on ES cell pluripotency. Alternatively, co-activator usage can also be regulated at the kinase level. One of these possibilities is regulation by the PI3K pathway, upon activation of this pathway the kinase AKT translocates to the nucleus where it can phosphorylate p300 (Huang and Chen, 2005). This phosphorylation decreases the β-catenin-p300 interaction and hence promotes self-renewal. This indicates that other signaling pathways can influence the outcome of Wnt signaling by modulating co-activator usage of β-catenin.

Although several reports contradict each other, it does not mean that only a few of them are true. Obviously Wnt signaling is not the only pathway involved in ES cell self-renewal. As there is a lot of crosstalk between pathways in ES cells, activation of one pathway may compensate for the loss of the other. Moreover, activities of other pathways influence the outcome of Wnt signaling. Most of the labs use different cell lines and culture conditions, suggesting that perhaps different signaling pathways are active in the studied ES cells (reviewed by Ohtsuka and Dalton, 2008). Next to this, being involved in ES cell self-renewal does not exclude the possibility that Wnt signaling is involved in the differentiation process. Wnt signaling is reported to be involved in many processes in many different organisms. It is very likely Wnt signaling is needed for both differentiation and self-renewal.

Dravid and colleagues showed that Wnt signaling alone could not block differentiation of hES cells in feeder-free conditions, yet Wnt signaling was able to promote self-renewal of these cells (Dravid et al., 2005). The same group came to a similar conclusion a few years later when using different Wnts to stimulate the Wnt signaling pathway (Cai et al., 2007). Together this suggests that Wnt signaling is involved in both regulation of differentiation and maintaining self-renewal in ES cells.
In conclusion there are a lot of reports that show involvement of Wnt signaling in either differentiation or self-renewal of both human and mouse ES cells. Differences in cell lines, culture conditions or duration of induction might contribute to these differences, or perhaps Wnt signaling is involved in both processes. Conflicting results about the role of β-catenin in ES cells might come from the different roles β-catenin can play in ES cells. It can either function as a co-activator or as a de-repressor of TCF3. Another possibility is that PP2A or PI3K/AKT-signaling regulates whether CBP or p300 acts as co-activator for β-catenin mediated transcription, which in turn might determine if Wnt signaling promotes self-renewal or induces differentiation.

Chapter 6 Conclusion

Wnt signaling is an important signal transduction pathway for regulation of self-renewal and differentiation in many cell types; active Wnt signaling promotes self-renewal in both hematopoietic and intestinal stem cells. The role of Wnt signaling in ES cells however remains a topic of debate. Several experiments have shown that activation of Wnt signaling, by either supplying ES cells with GSK3 inhibitors or Wnt ligands, promotes self-renewal. Some groups even found that Wnt signaling can sustain self-renewal in ES cells in the absence of other growth factors (Yi et al., 2011), whereas others found that additional signaling factors were needed as well (Singla et al., 2006; ten Berge et al., 2011). Wnt signaling also facilitates the reprogramming of fibroblasts to a pluripotent state, further suggesting that Wnt signaling is involved in regulation of pluripotency in ES cells. Several molecular explanations have been proposed for how Wnt signaling promotes ES cell self-renewal. TCF3, a Wnt regulated transcription factor, is an integral component of the core regulatory network of pluripotency in ES cells. This network consists of OCT4, SOX2 and NANOG, and TCF3 would act as an repressor of these transcription factors, as it binds in the same regions on promoters co-occupied by all four factors. Activation of Wnt signaling causes β-catenin to be stabilized and β-catenin subsequently localizes to the nucleus where it binds to TCF3 and relieves the repression on OCT4, SOX2 and NANOG. Aside from TCF3, TCF1/LEF1 might also play a role in the regulation of ES cell self-renewal and a direct interaction of β-catenin with OCT4 was proposed to be involved in the regulation of pluripotency as well.

On the other hand, other experiments showed a contrasting role for Wnt signaling in ES cells; active Wnt signaling resulted in differentiation of ES cells in various lineages, which is in sharp contrast with the previously mentioned results. These contradictory observations might be caused by differences in cell line, cell state or time of Wnt signaling activation. However, these apparently contrasting results do not necessarily exclude each other. It is possible that Wnt signaling promotes both self-renewal and differentiation in ES cells. Usage of either CBP or p300 as co-activator for β-catenin mediated transcription influences the outcome of Wnt signaling, CBP as co-activator promotes self-renewal while p300 as a co-activator initiates differentiation. Co-activator usage is determined by the phosphorylation state of p300 which is regulated by PP2A activity and extracellular signaling pathways (reviewed by Teo and Kahn, 2010). CBP and p300 recruit different co-factors to the promoters when bound to β-catenin, which results in differential regulation of self-renewal or differentiation.

There is currently no consensus whether Wnt signaling promotes self-renewal, differentiation or both, but the data suggests that Wnt signaling plays a dual role in regulation of these processes. Wnt signaling is not the only signaling pathway able to regulate ES cell self-renewal or differentiation, other signaling pathway such as the PI3K, LIF and BMP4 pathways play important roles in these processes as well (reviewed by Ohtsuka and Dalton, 2008). These other signaling pathways can influence the outcome of Wnt signaling on ES cell self-renewal and it would be wrong to look at Wnt signaling as the only signaling pathway regulating ES cell pluripotency. In contrast, regulation of self-renewal should be seen as a complex regulatory network involving different signaling pathways, and Wnt signaling plays an important role in this network. This makes Wnt signaling an important signaling pathway to study, especially since Wnt signaling does not only play a role in regulation of self-renewal of ES- and iPS cells, but in cancer cells as well. Future studies will expand our fundamental knowledge about the molecular mechanisms underlying the regulation of self-renewal in these different cells.

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