ABSTRACT

Leukemia inhibitory factor (LIF) is a member of the interleukin-6 (IL-6) cytokine family. All members of this family activate signal transducer and activator of transcription 3 (STAT3), a transcription factor that influences stem and progenitor cell identity, proliferation and cytoprotection. The role of LIF in development was first identified when LIF was demonstrated to support the propagation of mouse embryonic stem cells. Subsequent studies of mice deficient for components of the LIF pathway have revealed important roles for LIF signaling during development and homeostasis. Here and in the accompanying poster, we provide a broad overview of JAK-STAT signaling during development, with a specific focus on LIF-mediated JAK-STAT3 activation.

KEY WORDS: Leukemia inhibitory factor, STAT3, Interleukin-6 (IL-6) cytokine

Introduction

Embryonic stem cells (ESCs) – pluripotent cells derived from the mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981) – are an important model system for studying early development. Soon after the initial derivation of mouse (m) ESCs it was discovered that their in vitro propagation required the activity of the cytokine leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988), which functions by activating the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway (Boeuf et al., 1997; Niwa et al., 1998). Subsequent studies elaborated on the role of JAK-STAT signaling in mESCs and their derivatives, and meaningful insights into the role of the LIF JAK/STAT3 signaling axis in early development have also emerged. Whereas human (h) ESCs were shown not to depend on JAK-STAT signaling for their propagation (Beattie et al., 2005; Dahéron et al., 2004; Humphrey et al., 2004; Vallier et al., 2005), recent advances in our understanding of different human pluripotent
cell states (Buecker et al., 2010; Chan et al., 2013; Gafni et al., 2013; Hanna et al., 2010; Theunissen et al., 2014; Ware et al., 2014) have suggested a role for LIF signaling in human development. Here and in the accompanying poster, we provide an overview of LIF-JAK-STAT signaling and discuss how it regulates various stages of development.

**Mechanisms of JAK-STAT signaling**

JAK-STAT signaling is mediated primarily through the interleukin-6 (IL-6) family of cytokines that signal via receptors categorized as either non-signaling α-receptors or as signaling receptors. LIF, for instance, first binds to its signaling receptor, LIF-R (Gearing et al., 1991), and recruits another signaling receptor, glycoprotein 130 (GP130), to form a heterodimer that mediates downstream signal transduction. Both LIF-R and GP130, being signaling receptors, are used by other IL-6 family members. In contrast to LIF, however, other IL-6 family cytokines first bind to specific, low-affinity non-signaling receptor subunits (such as IL-6R, IL-11R, CT-1R and CNTFR-1) and initiate either the homodimerization of GP130 receptors (Hibi et al., 1990) or the heterodimerization of LIF-R and GP130. Additionally, the cytokine oncostatin M (OSM) is able to bind either LIF-R or its own specific signaling receptor, OSM-R, to form a heterodimer with GP130 [reviewed by Heinrich et al. (2003, 1998)]. Upon dimerization, the signaling receptors recruit and phosphorylate JAKs (JAK1, JAK2, JAK3 and Tyk2) which, in turn, phosphorylate STAT3 and other STAT family members (STAT1 and STAT5). The cascade culminates in the dimerization of phosphorylated STAT3 (pSTAT3), its translocation to the nucleus and the direct regulation of the transcription of a wide range of genes. Included in these target genes is the potent JAK-STAT inhibitor suppressor of cytokine signaling 3 (SOCS3) (Naka et al., 1997; Starr et al., 1997). Inhibition of STAT3 is also independently mediated through protein inhibitor of activated STAT3 (PIAS3) (Chung et al., 1997), thus forming a rapidly responding and reinforced feedback mechanism to regulate JAK-STAT signaling. Paradoxically, JAK-STAT signaling also acts in a feed-forward manner, initiating the rapid transcription of STAT3, JAK1, GP130 and LIF-R (Davey et al., 2007; He et al., 2005). These negative and positive autoregulatory aspects of JAK-STAT signaling are capable of cycling between dormancy and activity in response to specific developmental cues and in response to external stimuli postnatally.

JAK-STAT signaling also activates mitogen-activated protein kinases (MAPKs) (Burdon et al., 1999; Ernst et al., 1996), cyclic AMP-responsive element binding protein (CREB) and ribosomal s6 kinase (Boeuf et al., 2001), src family kinases (Ameren et al., 2004) and phosphatidylinositol-3-OH kinase (PI3K) (Niwa et al., 2009; Paling et al., 2004). These branches of signaling will not be reviewed here. Instead, we will focus on JAK-STAT activation by LIF and related cytokines.

**JAK-STAT signaling in the maintenance of pluripotency in vitro**

The existence of a pluripotent cell population in vivo is transient, as the pluripotent epiblast rapidly differentiates into the embryo proper and into components of extraembryonic tissue. The ability to maintain a pluripotent state in vitro in spite of this transience, as occurs in derivation of mESCs (Evans and Kaufman, 1981; Martin, 1981), is thus remarkable. Central to this maintenance of pluripotency is STAT3 activation; in its absence, mESCs are unable to self-renew (Boeuf et al., 1997; Niwa et al., 1998). Conversely, constitutive activation of STAT3 is able to confer LIF-independent self-renewal (Matsuda et al., 1999). Several other transcription factors, including Nanog (Chambers et al., 2003; Mitsui et al., 2003), KLF4, KLF (Bourillot et al., 2009; Hall et al., 2009; Niwa et al., 2009), GBX2 (Tai and Ying, 2013) and TFCP2L1 (Martello et al., 2013), can confer varying degrees of LIF-independent self-renewal. Importantly, all of these transcription factors, with the exception of Nanog, are direct targets of activated STAT3 (pSTAT3), underscoring the importance of JAK-STAT signaling in the maintenance of pluripotency. Additionally, the endogenous secretion of GP130 ligands in mESCs buffers cells against precocious differentiation (Davey and Zandstra, 2006) and, combined with the autoregulatory behavior of JAK-STAT signaling, supports robust propagation of mESCs. In the absence of exogenous LIF, however, the secretion of autocrine GP130 ligands is insufficient to maintain pluripotency in standard mESC conditions (Davey et al., 2007). A threshold of STAT3 activity is required to inhibit differentiation caused by components contained in media (e.g. serum) or by the endogenous secretion of differentiation-inducing fibroblast growth factor 4 (FGF4) (Kunath et al., 2007). Therefore, a precarious balance exists between the differentiation-inhibiting and differentiation-inducing signals that govern cell fate decisions of mESCs. Indeed, the suppression of FGF4 signaling by inhibition of both of its receptors (using the small molecular inhibitor SU5402) and of its downstream target ERK (using the MAPK inhibitor PD184352) is sufficient to maintain pluripotency (Ying et al., 2008). However, Ying and colleagues demonstrated that FGF blockade alone is unable to prevent precocious differentiation and apoptosis, and that the addition of a Wnt signaling agonist, CHIR99021, is required for LIF-independent propagation of mESCs. Together, PD0325901 (a MAPK inhibitor that is more specific than PD184352) and CHIR99021 make up ‘2i’ medium; this medium is able to robustly support mESC propagation, even in the complete absence of JAK-STAT signaling (e.g. in STAT3 null or STAT3−/− cells). Consistent with this, Wnt signaling has emerged as a crucial mediator of mESC pluripotency (ten Berge et al., 2011). Whereas these observations suggest the dispensability of JAK-STAT signaling in mESC propagation, the continual presence of its downstream target TFCP2L1 is required for the maintenance of pluripotency in already established pluripotent cells (Martello et al., 2013). Notably, Nanog lies downstream of TFCP2L1 and therefore forms a hierarchical core of the JAK-STAT signaling pathway, demonstrating its importance in the maintenance of pluripotency, even in the absence of several upstream effectors.

**JAK-STAT signaling in early development**

Whereas the primary role of typically LIF-activated JAK-STAT signaling in vitro is to maintain pluripotency, its roles in vivo are nuanced and varied. Knockout studies of individual JAK-STAT signaling components in mice have been used to elucidate these roles. Beginning upstream, it was shown that the knockout of LIF is not detrimental to the development of mice. Female LIF−/− mice, however, are sterile, due to the requirement of maternal LIF for blastocyst implantation (Stewart et al., 1992). Regardless, the full development of LIF knockout mice to adulthood demonstrates that the absence of LIF during development has no lethal effects. This result is most likely due to redundancies in signaling molecules, as OSM, cardiotophin 1 (CT-1) and ciliary neurotrophic factor (CNTF) are all able to signal through the same receptors. Consistent with this, knockouts of downstream effectors of LIF all result in either embryonic or perinatal death; death occurs earlier in the absence of effectors with fewer redundancies and/or more global effects. For example, the knockout of LIF-R results in death at birth
due to severe glial and motor neuron deficits (Li et al., 1995; Ware et al., 1995), whereas knockout of the global IL-6 family receptor, GP130, results in death between embryonic day (E) 12 and E18, with embryos exhibiting severe defects in cardiac, hematopoietic and neural development (Nakashima et al., 1999a; Yoshida et al., 1996). Additionally, Jak1−/− mice die perinatally, demonstrating a defect in lymphoid development (Rodig et al., 1998), whereas Jak2−/− mice die at E12 due to a failure in definitive hematopoiesis (Neubauer et al., 1998; Parganas et al., 1998). An absence of STAT3 results in the most severe of the phenotypes, with embryos dying soon after implantation (~E6.5) (Takeda et al., 1997). Owing to the emergence of STAT3 activity in the visceral endoderm (VE) coinciding with the time of death, Takeda and colleagues reasoned that the embryo starves as a result of the disruption of the VE and that this explains the STAT3 knockout phenotype. An alternative hypothesis is that STAT3 is required to form a pluripotent cell population. The zygotic STAT3 knockout model is insufficient to address this hypothesis, as it does not account for the ability of embryos to undergo diapause due to the lack of a pluripotent cell population. The zygotic STAT3 knockout model is insufficient to address this hypothesis, as it does not account for the ability of embryos to undergo diapause due to the lack of a pluripotent cell population.

Developmental progression requires loss of JAK-STAT signaling at the peri-implantation stage

Given that JAK-STAT signaling activity is sufficient to maintain pluripotency, which occurs at the expense of initiating differentiation, it follows that the embryo proper is required to suppress or overcome JAK-STAT signaling in order to progress through development. Indeed, whereas extraembryonic STAT3 expression is observed soon after implantation and during gastrulation (from E4.5 to E8.5), high levels of STAT3 expression in the embryo proper are not observed until E7.5 (Duncan et al., 1997), well after the initiation of gastrulation. While it remains unclear whether constitutively active STAT3 arrests development of the post-implantation embryo, the ability of constitutive STAT3 activity to maintain pluripotency in vitro and during diapause suggests that STAT3 activation hinders progression to gastrulation. An active suppression of JAK-STAT signaling has been observed during the differentiation of mouse ESCs to epiblast stem cells (EpiSCs) (Onishi et al., 2012), which are an in vitro model of post-implantation pluripotency and which do not require LIF for self-renewal (Brons et al., 2007; Tesar et al., 2007). Additionally, EpiSCs are unresponsive to LIF signaling (Onishi et al., 2014). Together, these data suggest an innate suppressive mechanism to ensure minimal STAT3 activation at the peri-implantation stage. This suppression might not be an absolute requirement, however, as the presence of LIF signaling is not dominant in preventing differentiation, whether precocious or directed. Consistent with this, differentiation to EpiSCs can occur in the presence of LIF (ten Berge et al., 2011), as can the differentiation to neural stem cells (Tropepe et al., 2001). Together, these data illustrate that the active suppression of JAK-STAT signaling in vitro is not an absolute requirement, due in part to the presence of non-physiological, high concentrations of cytokines that drive differentiation; however, it might be a requirement in vivo to ensure correct responses to a highly coordinated set of morphogens.

The recovery of JAK-STAT signaling post-implantation is required for early neural development

Soon after its loss in the epiblast, JAK-STAT signaling is present again during neural development, in which it is required in a stage-dependent manner to support the generation of mature glial cells. The absence of GP130, for example, was shown to result in severe, observable loss of both neurons and astrocytes (a subset of glia) at E18.5 (Nakashima et al., 1999a). Consistent with the loss of JAK-STAT signaling in the epiblast, early-stage (E10-11) cortical neural progenitor cells (NPCs) are repressed in responsiveness to LIF (He et al., 2005). By contrast, later-stage NPCs, isolated at E14 or continuously cultured in vitro following isolation at E10-11, respond robustly to LIF and require it to differentiate to astrocytes (Bonni et al., 1997; He et al., 2005). JAK-STAT signaling thus appears to serve a dual role in NPCs, as basal levels of activation in E14 NPCs promote self-renewal and suggest that JAK-STAT activation in neural stem/progenitor cells buffers them against differentiation (as in mESCs) (Shimazaki et al., 2001). This dual role is probably due to a switch-like change in responsiveness coupled with context-dependent signaling. Indeed, an autoregulatory JAK-STAT feed-forward loop that re-initiates signaling ensures a rapid, robust recovery from dormancy (Davey et al., 2007; He et al., 2005). However, to prevent precocious reactivation of LIF responsiveness, this re-initiation requires an additional initial cue: bone morphogenetic protein (BMP) signaling synergizes with LIF, specifically through formation of a downstream transcriptional complex (Nakashima et al., 1999b), to facilitate astrocyte generation. This complex is also present in EpiSCs and leads to the initial reactivation of LIF responsiveness (Onishi et al., 2014). Therefore, we predict that JAK-STAT signaling is awakened from its dormancy in a cascade that first requires specific cues (BMP and GP130 signaling) and then depends on a feed-forward autoregulatory loop to generate a switch-like response to mediate timing-sensitive differentiation programs. These cues might also direct the lineage commitment mediated by JAK-STAT signaling. Other lineages that involve JAK-STAT signaling during their development might also depend on this mechanism to reinitiate signaling.

LIF signaling in later development

JAK-STAT signaling is observed broadly in post-implantation development, and its absence manifests itself in defects in neural, cardiac and hematopoietic lineages. Conditional knockouts of...
STAT3, which bypass the early lethality of pan-knockout STAT3 mice, further illuminate the role of JAK-STAT signaling during development [reviewed by Levy and Lee (2002)]. However, examination of these data demonstrates that JAK-STAT signaling is not an absolute requirement for the development of most tissues. Whereas the embryonic lethality and associated cardiac defects of gp130−/− mice (Yoshida et al., 1996) suggest that JAK-STAT signaling is indispensable for cardiac development, the conditional knockout of STAT3 in cardiac cells does not result in embryonic lethality or the absence of cardiomyocytes. Instead, STAT3-null cardiomyocytes demonstrate both an inability to protect (buffer) against apoptosis and the upregulation of pro-inflammatory TNF-α signals upon lipopolysaccharide-induced inflammation (Jacoby et al., 2003). Consistent with this, JAK-STAT signaling has known roles in protecting the heart from stress-induced injury by directly regulating genes involved in proliferation and cytoprotection [reviewed by Fischer and Hilfiker-Kleiner (2007)]. Specifically, STAT3 regulates the expression of factors implicated in hypertrophy (c-fos, ANP), angiogenesis (VEGF) and cytoprotection (MnSOD, Bcl-xl) [reviewed by Jacoby et al. (2003)]. Similarly, in hematopoietic cells, the absence of STAT3 does not lead to ablation of cells but rather a reduction in cytoprotective and anti-apoptotic capacity. For example, T-cells devoid of STAT3 demonstrate no defects in development but instead show loss of proliferative capacity in response to IL-6 and are more sensitive to apoptosis (Takeda et al., 1998). Additionally, conditional STAT3 knockout in keratinocytes results in impaired wound healing and hair cycling (Sano et al., 1999), whereas knockout in the liver results in impaired acute-phase response (Alonzi et al., 2001). In these examples, the multiple pathologies can be related to a lack of transcriptional regulation of the respective, tissue-specific STAT3 target genes. Taken together, these findings suggest that later in development, JAK-STAT signaling plays a role in response to injury in restricted cell types rather than in developmental processes, a role well suited to JAK-STAT signaling due to its ability to rapidly recover from dormancy and to its direct regulation of many cytoprotective genes.

**JAK-STAT signaling during germ line development**

Primordial germ cells (PGCs) are the early embryonic precursors to adult germ cells and gametes. The initial specification of PGCs from within the posterior, proximal epiblast (Ohinata et al., 2005) is driven by supportive BMP signals emanating from the extraembryonic ectoderm (ExE) and antagonistic BMP suppression from the anterior visceral endoderm (AVE) (Lawson et al., 1999; Umulis et al., 2009). Although much of the focus of signals governing PGC specification has centered on BMP, JAK-STAT signaling has also emerged as an important factor that drives the acquisition and maturation of PGC cell fate. Evidence of its role in mouse PGC specification is found in vitro, during the directed differentiation of either cultured mouse pluripotent stem cells (PSCs) or explanted E6.0 epiblasts to PGCs (Hayashi et al., 2011; Ohinata et al., 2009). In both cases, BMP alone is required for the initial expression of the PGC precursor marker, Blimp1, and of markers for committed PGCs, Stella or alkaline phosphatase (ALP). However, the emergence of PGC-like cells in vitro is enhanced by the presence of LIF, and is optimal in the presence of a cocktail of cytokines, including LIF, BMP8b, stem cell factor (SCF, also known as steel factor) and epidermal growth factor (EGF). Rigorous analysis is currently lacking on the role of JAK-STAT signaling during the specification of PGC fate in vivo, and as such, conclusions about its role in this cell fate decision cannot be made. However, using cues from other signaling systems, in vitro work and other organisms, it appears that JAK-STAT activation enhances the motility, proliferation and survival of PGCs. SCF, which signals through STAT3, is a known survival factor and potential mitogen for PGCs in vitro (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991), and it is crucial for PGC motility in vivo (Gu et al., 2009). However, whether STAT3 is the primary mediator of the actions of SCF remains an area of active investigation. A similar observation of compromised motility is made in JAK-STAT signaling-deficient Drosophila melanogaster PGCs (Brown et al., 2006). Whereas its role in motility in mammalian systems remains unclear, JAK-STAT signaling might act on PGCs as a mitogen or survival factor. Indeed, GP130 signaling has been shown to drive the expansion of PGCs in vitro (Koshimizu et al., 1996; Matsui et al., 1991). As conditional knockouts for individual JAK-STAT signaling components, specifically those downstream in signaling, in PGCs are currently limited, it remains an open question whether known mitogens and survival factors converge on JAK-STAT signaling to exert its functions. Nevertheless, JAK-STAT signaling remains an important component during the development of PGCs.

**JAK-STAT signaling in the re-establishment of pluripotency**

PGCs themselves are unipotent, capable of only generating adult gametes. Nevertheless, PGCs are capable of being reprogrammed back to a pluripotent state given specific cues. This reprogramming was first observed when PGCs were identified as a source cell of teratocarcinomas (Stevens, 1967). PGCs also give rise to teratomas and teratocarcinomas when ectopically implanted (Stevens, 1970). This ability to regain pluripotency when ectopically stimulated can be recapitulated in vitro: the addition of a combination of LIF, SCF and basic fibroblast growth factor (bFGF) is sufficient to reprogram PGCs, albeit at a low frequency, to pluripotent embryonic germ cells (EGCs) (Matsui et al., 1992; Resnick et al., 1992). The addition of LIF or other GP130 ligands is an absolute requirement for this transformation (Koshimizu et al., 1996). LIF, specifically, is required in the later stages of EGC conversion, and STAT3 activation during this time is also an absolute requirement (Leitch et al., 2013). Similarly, reprogramming to pluripotency can occur in unipotent germline stem cells (GSCs) derived from neonatal (Kanatsu-Shinohara et al., 2004) and adult testes (Ko et al., 2009), although the role of JAK-STAT signaling in these events remains unclear. JAK-STAT signaling therefore demonstrates a remarkable ability to drive the reacquisition of pluripotency in closely related cell types. Thus, although it does not appear to have an essential role in establishing pluripotency immediately after fertilization (i.e. during the totipotency-to-pluripotency transition, from zygote to blastocyst), STAT3 activation has a central role in ectopically re-establishing pluripotency from committed germ cells. Consistent with this, STAT3 activation is sufficient to drive reprogramming to naïve pluripotency, both in EpiSCs (Bao et al., 2009; Onishi et al., 2012; Yang et al., 2010) and in partially reprogrammed induced pluripotent stem cells (iPSCs) (Yang et al., 2010). Whereas these examples occur in the context of in vitro culture or in vivo malignancies, JAK-STAT signaling does appear to maintain an important role in normal germline development following PGC specification. This has been shown by its requirement to specify the male germ line in Drosophila (Sheng et al., 2009), although it remains unclear whether this role is conserved in mammals.

**Discussion**

In summary, JAK-STAT signaling is present in many different cell types and its activation regulates diverse physiological responses. In early development, the activation of JAK-STAT signaling during diapause ensures the maintenance and therefore protection of the
embryo in sub-optimal conditions. Outside of this optional, protective feature, JAK-STAT signaling is not required in the epiblast. In fact, its continuous activity prevents the exit from pluripotency and thus prevents progression to gastrulation. Paradoxically, its complete absence from the embryo is also detrimental and leads to the death of the embryo. However, this most likely occurs through a secondary effect – the compromising of extraembryonic tissue – and JAK-STAT signaling is probably not required in the embryo proper at the initiation of gastrulation. Following gastrulation, JAK-STAT signaling re-emerges, both in the germ line and in the development of some somatic tissues. However, JAK-STAT signaling does not appear to be a requirement for the development of these tissues, as conditional knockouts of STAT3 surprisingly result in mild phenotypes. Instead, the absence of JAK-STAT signaling later in development results in aberrant responses to perturbations or stress. Indeed, as a whole, a picture emerges in which on the one hand JAK-STAT activation serves to protect stem cells during development from overt differentiation inducing signals. On the other hand, in more differentiated cells (emerging later in development or during homeostasis), it acts to buffer cells against other types of signals, such as apoptosis or proliferation.

Owing to its role as a master regulator of the pluripotency-related transcriptional network, dysregulated, hyper-active JAK-STAT signaling can lead to malignant reacquisition of pluripotency in the form of teratocarcinomas. As such, and possibly as an evolutionary prevention of the development of this malignancy, JAK-STAT signaling is muted in pluripotent stem cells derived from many different species, including most strains of mice (non-permissive strains), and is unable to maintain their pluripotency (Brons et al., 2007; Park et al., 2013; Thomson et al., 1998, 1995; Wang et al., 2008). Indeed, the first mESC lines were derived from strains of mice (129) that spontaneously generate teratocarcinomas (Stevens and Little, 1954). Only with appropriate co-stimulation is JAK-STAT signaling able to maintain the pluripotency of cells derived from non-permissive mouse strains (Hanna et al., 2009), rats (Buehr et al., 2008) and, importantly, humans (Chan et al., 2013; Gafni et al., 2013; Hanna et al., 2010; Theunissen et al., 2014; Ware et al., 2014). Together, this suggests that JAK-STAT signaling plays a conserved role across different species to ensure the preservation of pluripotency. In an extension to human pluripotency, apparently pluripotent stem cell lines with some properties reflective of different stages of mouse pluripotency can now be propagated, including under conditions supported by JAK-STAT signaling (Chan et al., 2013; Gafni et al., 2013; Hanna et al., 2010; Theunissen et al., 2014; Ware et al., 2014). This paradigm shift suggests that the numerous studies conducted in mice concerning the role LIF in development, are, at least in part, relevant to human development. Excitingly, a recent demonstration of this relevance comes with the derivation of human PGCs from LIF-responsive hESCs (Irie et al., 2015).

Acknowledgements
We thank Joel Ostblom for insightful discussions and critical comments on the manuscript and poster.

Competing interests
The authors declare no competing or financial interests.

Funding
We acknowledge the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada (PWZ Discovery) for funding of this work. P.W.Z. is the Canada Research Chair in Stem Cell Bioengineering.

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Hall, J., Guo, G., Wray, J., Eyres, I., Nichols, J., Grotewold, L., Morfopoulou, S., Koshimizu, U., Taga, T., Watanabe, M., Saito, M., Shirayoshi, Y., Kishimoto, T., Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Mu...