Cytoplasmic mislocalization of p27 and cdk2 mediates the anti-migratory and anti-proliferative effects of Nodal in human trophoblast cells

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Running title: p27 and cdk2 mediate Nodal actions
Key words: p27, cdk2, migration, invasion, Nodal, ALK7

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Summary

p27Kip1, a cyclin-dependent kinase (CDK) inhibitor, is a multi-functional protein that regulates various cellular activities. Trophoblast proliferation, migration, and invasion are some of the key processes of placental development. We have recently reported that Nodal, a member of the transforming growth factor-β (TGF-β) superfamily, inhibits human trophoblast cell proliferation, migration and invasion. In this study, we investigated the mechanism by which Nodal regulates trophoblast activities. We found that Nodal increased p27 mRNA and protein levels by enhancing their stability. Interestingly, Nodal signaling also induced nuclear export of p27 and cdk2. Cytoplasmic translocation of p27 induced by Nodal requires p27 phosphorylation at S10. In addition, Nodal enhanced the association of p27 with cdk2, cdk5 and a microtubule destabilizing protein; stathmin, and induced stathmin phosphorylation at S25 and S38. Furthermore, Nodal increased tubulin stability as revealed by immunofluorescent staining of acetylated tubulin. Finally, silencing of p27 reversed the inhibitory effect of Nodal on trophoblast cell proliferation, migration, and invasion. Taken together, our findings revealed a novel function of simultaneous p27 and cdk2 cytoplasmic mislocalization in mediating growth factor-regulated cell proliferation, migration and invasion.
Introduction

Trophoblast proliferation, migration and invasion are critical for normal placental development and therefore successful pregnancy. During pregnancy, trophoblast cells proliferate, differentiate, and invade the uterine myometrium to form a functional placenta. The development and function of the placenta are regulated by an extensive array of cytokines, growth factors and hormones, including members of the transforming growth factor-β (TGF-β) superfamily (Caniggia et al., 2000a). We have previously reported that Nodal, a member of the TGF-β superfamily, is expressed in human placenta (Roberts et al., 2003) and exerts inhibitory effects on trophoblast cell proliferation, migration and invasion while induces apoptosis (Munir et al., 2004; Nadeem et al., 2011).

The role of p27 in inhibiting cell cycle progression has been well established. It inhibits G1 cyclin-Cdk complexes that prevent phosphorylation of retinoblastoma protein (Rb), leading to subsequent inhibition of E2F dependent transcriptions of S-phase regulatory proteins (Toyoshima and Hunter, 1994). In addition, there is considerable evidence that p27 also plays an important role in other cellular processes, such as differentiation, migration and apoptosis (Sgambato et al., 2000). It is generally accepted that p27 nuclear localization is of prime importance for the control of cell cycle progression (Boehm et al., 2002); however, p27 can shuttle from the nucleus to the cytoplasm (Blain et al., 2003; Reed, 2002), suggesting that the same protein exerts additional activities in different subcellular compartments. Cytoplasmic p27 has been reported to interact with stathmin (a microtubule destabilizing protein) and RhoA (a member of Rho GTPase family) to inhibit and stimulate cell migration, respectively (Baldassarre et al., 2005; Besson et al., 2004).

Several phosphorylation sites of p27 have been recognized which determine its activity or fate in the cell. Among them, phosphorylation at S10 is considered to be responsible for its export from the nucleus, phosphorylation at T157, T170, T197, and T198 restricts p27 to the cytosol, while phosphorylation at T187 targets it for degradation (Fujita et al., 2003b; Ishida et al., 2000; Morisaki et al., 1997; Rodier et al., 2001; Short et al., 2010). Several kinases have been reported to phosphorylate p27 at S10; such as Cdk5 in neurons (Kawauchi et al., 2006), Mirk/dyrk1B in quiescent cells (Deng et al., 2004), hKIS (Connor et al., 2003), PKB/AKT (Liang et al., 2002) and ERK2 (Rodier et al., 2001) during G1/S transition, JNK in glioma cells (Mielke, 2008), and CAMKII in HeLa cells (Kajihara et al.). AKT phosphorylates p27 at T157 and promotes its interaction with 14-3-3 protein, resulting in its cytoplasmic sequestration (Liang et al., 2002;
Phosphorylation of p27 at T198 enhances its stability and sequestration by AKT, RSK1 and RSK2 (Fujita et al., 2002; Fujita et al., 2003a; Motti et al., 2005). CyclinE/cdk2 phosphorylates p27 at T187 and makes it a target of the ubiquitin-proteasome degradation pathway (Montagnoli et al., 1999).

We have previously shown that Nodal upregulates p27 mRNA levels in trophoblast cells (Munir et al., 2004). In this study, we further examined the regulation of p27 by Nodal signaling. We found that Nodal increased p27 levels through multiple mechanisms. Interestingly, Nodal also induced p27 and cdk2 cytoplasmic translocation, which leads to inhibition of cell proliferation, migration and invasion.

Results

1. Nodal and ALK7 upregulate p27 expression through multiple mechanisms:

Previously, we have reported that overexpression of Nodal or constitutively active ALK7 (ALK7-ca) increased p27 mRNA levels in trophoblast cells (Munir et al., 2004). To confirm this finding, we first treated cells with recombinant Nodal (rNodal) and observed that rNodal significantly increased p27 mRNA levels as compared to the control (Fig. 1A). To determine if Nodal increases the stability of p27 mRNA, cells were treated with Actinomycin D (5 µg/ml) in the presence or absence of rNodal. At 80 to 160 min after treatment, rNodal significantly increased p27 mRNA levels (Fig. 1B). Correspondingly, p27 protein levels were increased after rNodal treatment (suppl Fig. 1) or following transient transfection of Nodal and ALK7-ca constructs (Fig. 1C) resulting in the overexpression of Nodal and ALK7-ca, respectively (suppl Fig. 2). To examine if Nodal affects p27 protein stability, cells were treated with cycloheximide (CHX, 5 µg/ml), either alone or with rNodal. The p27 level was almost undetectable at 8 and 12 h post CHX treatment in the control group, while in the rNodal-treated group, p27 remained detectable for the entire duration of treatment (Fig.1D). Since it is documented that p27 can be degraded via the proteasome pathway through T187 phosphorylation (Vlach et al., 1997), we tested T187 phosphorylation status of p27. It was found that in cells treated with rNodal, phospho- p27-T187 level was decreased (Fig. 1E).

2. Nodal/ALK7 signaling induces p27 and cdk2 nuclear export:
To further analyze p27 regulation by Nodal, we examined p27 localization in cells transfected with control or Nodal-expressing constructs. Surprisingly, we observed that in cells transfected with Nodal plasmids encoding Nodal precursor (N53) or mature Nodal (N56), p27 was mainly detected in the cytoplasm, whereas in cells transfected with the control plasmid, p27 was mostly restricted to the nucleus (Fig. 2A). Time course studies revealed that p27 nuclear export occurred as early as 12 h after Nodal transfection (suppl. Fig. 3). Since Nodal inhibits trophoblast proliferation (Munir et al., 2004) and p27 is known to form a complex with cdk2 to block the G1/S progress (Toyoshima and Hunter, 1994), we examined the subcellular localization of cdk2 following Nodal transfection. Similar to p27, cdk2 was mainly detected in the nucleus in the control cells but Nodal overexpression resulted in its nuclear export (Fig. 2A).

It is known that Nodal acts through two type I receptors, ALK4 and ALK7 (Reissmann et al., 2001) and that TGF-β activates ALK5 while Activin-A binds to ALK4 to induce Smad2/3 phosphorylation (Graham and Peng, 2006), we tested whether Activin-A, TGF-β1, and their receptors have similar effects on p27 and cdk2 nuclear export as Nodal. We found that treatment with rNodal or TGF-β1, but not Activin-A, induced p27 and cdk2 nuclear export (Fig. 2B). Transfection with constitutively active ALK7 or ALK5 also resulted in the mislocalization of p27 and cdk2 in the cytoplasm (Fig. 2C). In contrast, constitutively active ALK4 failed to induce the cytoplasmic translocation of p27 and cdk2 (Fig. 2C).

3. Phosphorylation of p27 at S10 is required for Nodal-regulated p27 nuclear export:

It has been reported that S10 phosphorylation is one of the modifications required for p27 nuclear export (Rodier et al., 2001). To examine the phosphorylation status of p27 in trophoblast cells stimulated with rNodal, cell fractionation was performed, followed by immunoblotting with an antibody against phosphor-p27-S10. At earlier time points, rNodal increased phosphor-p27-S10 levels in the nuclear fraction (Fig. 3A) while at a later time point, an increase in p27-S10 accumulation in the cytoplasm was observed (Fig. 3B). Phosphorylation of p27 at T198 and T157 have been reported to cause cytoplasmic retention of p27 (Larrea et al., 2009b). Therefore, we also checked the level of p27-T198 and p27-T157. Nodal treatment decreased p27 phosphorylation at T157 and T198 (Fig. 3B). Again, p27-T187 level was decreased by Nodal (Fig. 3B).
To confirm that S10 phosphorylation is the prime protein modification required for its nuclear exclusion in Nodal signalling, cells were transfected with HA-tagged wild type p27 (WT), p27-S10A (a non-phosphorylation mutant), or p27-S10D (a phospho-mimetic mutant), and co-transfected with a Nodal expression plasmid or its control vector for 24 h. Double immunofluorescent staining was performed using anti-HA to monitor the transfected cells, and anti-p27 to determine the subcellular localization of p27. Nodal overexpression induced the nuclear export of p27-WT, but not p27-S10A (Fig. 3C). However, p27-S10D was detected in the cytoplasm with or without Nodal overexpression (Fig. 3C).

4. p27 plays a key role in Nodal–inhibited cell proliferation, migration and invasion:

Induction of p27 expression and p27/cdk2 cytoplamic localization by Nodal suggests that p27 mediates Nodal-induced cell cycle arrest. To test this, we performed siRNA mediated knockdown of p27 and cdk2 and then treated with rNodal. Efficiency of p27 and cdk2 siRNAs was verified by Western blotting (Fig. 4A). Treatment with rNodal decreased cell number in control siRNA-transfected cells. However, rNodal had no effect on cell growth (Fig. 4B) and cell viability (Fig. 4C) in cells transfected with p27 siRNA. Knockdown of cdk2 resulted in a significant decrease in cell number with or without rNodal treatment (Fig. 4B, D). Cdk2 knockdown also decreased cell viability as observed in WST-1 assays but it did not reverse the effect of rNodal (Fig. 4C).

Since it has been reported that cytoplasmic p27 regulates cell motility (Reed, 2002), we hypothesized that p27 is the key mediator of Nodal-regulated cell migration and invasion. To test this possibility, we silenced p27 and performed cell migration and invasion assays in control and rNodal-treated cells. In transwell cell migration assays, treatment with rNodal decreased the number of migrated cells in control siRNA-transfected cells. However, the effect of rNodal was completely reversed by p27 siRNAs (Fig. 5A). Interestingly, silencing of p27 decreased cell invasion; however, it also attenuated the inhibitory effect of Nodal on cell invasion (Fig. 5B).

5. Nodal promotes p27 interaction with stathmin to inhibit cell migration:

To explore the mechanism by which cytoplasmic p27 mediates Nodal-inhibited cell proliferation and migration, we examined the interacting proteins of p27 using immunoprecipitation. In p27-immunoprecipitated samples, we found that Nodal increased the
association of p27 with cdk2, cyclin E, cdk5 and stathmin (Fig. 5A). On the other hand, there was a decrease in p27/RhoA and p27/cyclin A complexes in rNodal-treated cells, when compared with the control (Fig. 6A). Similarly, immunoprecipitation using an antibody against stathmin showed that Nodal increased the association between stathmin and p27, as well as stathmin and cdk2/cdk5 (Fig. 6B).

Stathmin depolymerizes microtubules by sequestering α/β-tubulin and by enhancing microtubule catastrophe (Cassimeris, 2002), thereby enhancing cell motility (Baldassarre et al., 2005; Belletti et al., 2008). It has been reported that phosphorylation of stathmin reduces its microtubule destabilizing activity (Larsson et al., 1997). Therefore, we examined the phosphorylation status of stathmin in control and rNodal-treated cells following immunoprecipitation with the anti-stathmin antibody. Immunoblotting with phospho-specific stathmin antibodies showed that Nodal increased phosphorylation of stathmin at S25 and S38 (Fig. 6C). Interestingly, this effect of Nodal was decreased in the presence of a cdk2/cdk5 inhibitor (Fig. 6C).

Acetylated α-tubulin is often used as a marker for polymerized tubulin and therefore microtubule stability (Piperno et al., 1987). Histone deacetylase 6 (HDAC6) has been reported to deacetylate α-tubulin (Hubbert et al., 2002). To further explore the possibility that Nodal inhibits trophoblast cells by regulating the stability of microtubules, we assessed tubulin acetylation by immunofluorescence in control and Nodal-overexpressing cells. Nodal strongly increased acetylated α- tubulin levels (Fig. 7A). In addition, when cells were treated with tubacin, an inhibitor of HDAC6, α-tubulin acetylation was inhibited (Fig. 7B) and cell migration was strongly suppressed (Fig. 7C).

Discussion

We have previously shown that Nodal and ALK7 are expressed in human placenta (Roberts et al., 2003) and that Nodal signalling through ALK7 inhibits trophoblast cell proliferation (Munir et al., 2004), migration and invasion (Nadeem et al., 2011). In this study, we provided evidence that p27 is a key mediator of Nodal signalling in trophoblast cells. We found that Nodal not only upregulated p27 expression, but also induced p27 and cdk2 translocation to the cytoplasm, leading to inhibition of cell proliferation and migration/invasion.
The present study demonstrates that Nodal upregulates p27 mRNA and protein levels through multiple mechanisms. First, we found that in the presence of a transcription inhibitor, Nodal slowed down the decay of p27 mRNA. Second, we observed that Nodal increased p27 levels over a long period of time and this effect was still evident when protein translation was inhibited, suggesting that Nodal increases the p27 protein stability. This notion is further supported by the finding that Nodal decreased p27 phosphorylation at T187. Finally, Nodal induced p27 phosphorylation at S10, which has also been reported to increase p27 protein stability (Ishida et al., 2000). Thus, Nodal employs a variety of mechanisms to enhance and maintain p27 levels in trophoblast cells.

The most interesting and novel finding of this study is that Nodal induced nuclear export of p27 but despite the mislocalization of p27 to the cytosol, the cell cycle inhibitory function of p27 can still be preserved. Previous studies have suggested that subcellular localization of p27 is critical for G1-S-phase progression. In G0 phase, p27 is located exclusively in the nucleus inhibiting the kinase activity of cdk2. When cells progress to the G1 phase, p27 starts to move to the cytosol, rendering cdk2 active in the nucleus. Furthermore, a rapid decline of its level occurs due to increased proteolysis in the G1 phase and p27 expression level is minimal in the S-phase (Chu et al., 2008). In this study, we observed that upon Nodal treatment or overexpression, p27 is transported out to the cytoplasm. However, this did not lead to S-phase progression or p27 degradation as reported by others. Instead, we observed that in Nodal treated cells, there was accumulation of p27 in the cytoplasm, and consistent with our previous report that Nodal induced G1 cell cycle arrest in trophoblast cells (Munir et al., 2004), a decrease in cell proliferation. Our finding that silencing of p27 expression significantly reduced the inhibitory effect of Nodal on cell proliferation indicates that p27 mediates the antiproliferative effect of Nodal. We believe that the reason that p27 can still perform its antiproliferative function despite being transported out to the cytosol is because of the concomitant nuclear export of cdk2. It is generally accepted that cdk2 promotes G1/S transition by phosphorylating and thereby inactivating Rb, resulting in the activation of E2F transcription factors (Caillava and Baron-Van Evercooren, 2012). As these events take place in the nucleus, cytoplasmic mislocalization of cdk2 would render it inactive in promoting cell cycle progression. Nuclear export of cdk2 into the cytoplasm has been shown to mediate the anti-proliferative effect of 1,25-(OH)2 vitamin D3 in prostate cancer cells (Yang and Burnstein, 2003).
Three phosphorylation sites of p27, S10, T157 and T198, are important for its cytoplasmic localization (Fujita et al., 2003a; Ishida et al., 2000). We found that Nodal signaling induced p27 phosphorylation at S10 but decreased the level of T157 and T198 phosphorylation. In addition, we showed that a phosphorylation defective mutant of S10 was unable to undergo nuclear export upon Nodal treatment. These findings demonstrate that Nodal-induced p27 translocation is dependent on S10 phosphorylation of p27. Several kinases have been reported to phosphorylate p27 at S10, including Mirk/dyrk1B, hKIS, and cdk5 and CaMKII (Kajihara et al., 2010). Whether or not Nodal activates some of these kinases to phosphorylate p27 at S10 remains to be investigated. Similarly, how Nodal induces cdk2 translocation to the cytoplasm will be studied in the future.

We discovered several interacting proteins of p27 in the cytosol when cells were stimulated with Nodal. Specifically, we found that Nodal signaling enhanced p27 association with cdk2, cyclin E, cdk5, and stathmin. Recently it was reported that a decrease in p27-T198 phosphorylation promotes p27 binding to cdk2/cyclinE complexes (Kossatz et al., 2006). Consistent with this finding, we have also found that Nodal decreased p27 T198 phosphorylation and enhanced the association between p27 and cdk2/cyclin E. On the other hand, the association of p27/stathmin/cdk5 may be important for Nodal-inhibited cell migration and invasion. It has been reported that p27 can interact with stathmin to inhibit cell migration in fibrosarcoma and normal mouse fibroblasts (Baldassarre et al., 2005). Stathmin is a cytosolic protein that inhibits microtubule stability by sequestering tubulin heterodimers (Curmi et al., 1999) and by increasing the microtubule plus end catastrophe rate (Howell et al., 1999). Activity of stathmin is negatively regulated by phosphorylation of four serine residues; S16, S63, S25 and S38 thereby preventing its binding to tubulin (Di Paolo et al., 1997). Cdk5 and MAPK phosphorylate stathmin at S38 (Beretta et al., 1993; Hayashi et al., 2006) while cdk1 and cdk2 phosphorylate S25 (Brattsand et al., 1994; Kim et al., 2005). Interestingly, we found that Nodal enhanced the association of p27 with stathmin/cdk2/cdk5 and increased phosphorylation of stathmin at S38 and S25. The Nodal-induced stathmin phosphorylation was inhibited by a cdk2/cdk5 inhibitor, supporting the role of cdk2/cdk5 in regulating stathmin activity. The role of tubulin stabilization in trophoblast cell migration was further supported by the findings that Nodal enhanced α-tubulin acetylation and that inhibition of α-tubulin deacetylation by a HDAC6 inhibitor resulted in suppression of cell migration. Thus, it is possible that Nodal signaling triggers the export of p27 and cdk2, resulting
in the association of p27/cdk2/cdk5/stathmin to allow hyperphosphorylation and inactivation of
stathmin, leading to tubulin stabilization and thereby decreasing cell migration and invasion (Fig.
7D). More studies are required to further characterize how microtubule stability contributes to
Nodal-regulated cell migration.

The siRNA-mediated knockdown of p27 provided clear evidence that p27 is involved in
the inhibitory effect of Nodal on cell migration and invasion. Interestingly, invasion was
inhibited in cells transfected with p27 siRNA, suggesting that an increase level of p27 may
promote cell migration. However in the presence of Nodal, p27 siRNA reversed the inhibitory
effect of Nodal in cell migration and invasion. These results suggest that it is the modification,
not the level, of p27 induced by Nodal that is important for its anti-migratory activity. Indeed,
cytoplasmic p27 has been reported to inhibit or stimulate cell migration by interacting with
different proteins. For example, while p27 interaction with stathmin results in an inhibition of
cell migration (Baldassarre et al., 2005), p27 can also promote cell migration by interacting with
RhoA to inhibit its function (Besson et al., 2004; Larrea et al., 2009b). It has been reported that
phosphorylation of p27 at T198 promotes cytoplasmic mislocalization of p27 and enhances the
interaction between p27 and RhoA, leading to an increase in cell motility (Larrea et al., 2009a).
In this study, we found that Nodal inhibited p27 phosphorylation at T198 and the association
between p27 and RhoA. Thus, it appears that one of the mechanisms by which Nodal inhibits
trophoblast cell migration and invasion is through differential modulation of p27 phosphorylation
and its interaction with other cytoplasmic proteins. Nodal induces p27 phosphorylation at S10
and promotes p27-S10 binding to stathmin to inhibit cell migration. On the other hand, Nodal
also inhibits p27 phosphorylation at T198 and its binding to RhoA. The precise contribution of
the RhoA pathway to Nodal-inhibited migration and invasion requires further clarification.

Consistent with our previous observations that Nodal acts through ALK7 to inhibit
trophoblast cell proliferation (Munir et al., 2004), migration, and invasion (Nadeem et al., 2011),
we found that constitutively active ALK7 mimics the effect of Nodal on inducing p27 nuclear
export. Although ALK4 is a major receptor for Nodal during embryogenesis (Reissmann et al.,
2001), ectopic expression of constitutively active ALK4 did not induce p27/cdk2 export. ALK4
is also a receptor for activin, which is known to promote trophoblast cell invasion (Caniggia et
al., 1997). On the other hand, TGF-β1 and its receptor ALK5, which inhibits trophoblast cell
proliferation and invasion (Graham et al., 1994), also induced p27 and cdk2 translocation to the
cytoplasm. These findings suggest that concomitant mislocalization of p27 and cdk2 in the cytoplasm may be a common feature of growth factor-inhibited cell proliferation and cell migration in trophoblasts.

Abnormal placental development, including shallow invasion of trophoblast cells into the uterus and reduced trophoblast proliferation, has been linked to pregnancy-related disorders, such as preeclampsia (McMaster et al., 2004). We have recently reported that Nodal is overexpressed in preeclamptic placenta, suggesting that abnormal Nodal signaling may contribute to the pathogenesis of preeclampsia (Nadeem et al., 2011). Similarly, TGF-β signaling is also upregulated in preeclampsia (Caniggia et al., 2000b). It would be interesting to determine if p27 and cdk2 mislocalization, as well as stathmin impairment, are involved in the defective placentation and pregnancy complications.

Studies in mouse have suggested that Nodal plays critical roles in trophoblast differentiation and placental development. In mouse trophoblast stem cells (TSC), Nodal acts synergistically with fibroblast growth factor to maintain self-renewal and inhibit the precocious differentiation of TSC (Guzman-Ayala et al., 2004). A hypomorphic mutation of nodal gene in mice resulted in abnormal placenta development with an expansion of the invasive giant cells and spongiotrophoblast layers and a decrease in labyrinthine development (Ma et al., 2001). This, together with in vitro studies, suggests that Nodal inhibits differentiation into the giant cells pathway. Our findings that Nodal inhibits trophoblast migration and invasion suggest that Nodal may inhibit the differentiation into the invasive extravillous pathway. However, more work needs to be done, especially those using primary cultures of trophoblast cells and various markers of differentiation, to fully understand the role of Nodal in trophoblast differentiation during human placental development. Recently, human TSCs were identified in the placenta chorion (Genbacev et al., 2011). These TSCs will provide excellent models to study how trophoblast cell differentiation is controlled.

In summary, we have unveiled a key molecular mechanism by which Nodal regulates trophoblast cell proliferation and migration. Based on the observations that: 1) Nodal upregulates p27 expression at multiple levels; 2) Nodal induces p27- S10 phosphorylation and nuclear export of p27 and cdk2; and 3) Nodal promotes formation of complexes between p27 and stathmin, cdk2, and cdk5; and 4) silencing of p27 reverses the effect of Nodal on trophoblast cell
proliferation, migration and invasion, we conclude that cytosolic mislocalization of p27 and cdk2 mediates the anti-proliferative and anti-migratory/invasive effects of Nodal in trophoblast cells.

Materials and Methods

Cell line and cell culture: Immortalized human trophoblast cell line, HTR-8/SVneo, established from first trimester normal human trophoblast cells (Graham et al., 1993), was obtained from Dr. Graham (Queen’s University, ON, Canada) and cultured as described previously (Nadeem et al., 2011). Briefly, the cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO2. All the reagents for cell culture were purchased from Invitrogen Canada Inc.

RNA extraction, RT-PCR and qPCR: Total RNA was extracted from cells using TRIZOL reagent, as described previously (Xu et al., 2004) and stored at –80 °C until RT-PCR analyses. Primers used to amplify p27 and GAPDH have been previously published (Munir et al., 2004). Real-time PCR was carried out using Ssofast EvaGreen real-time PCR mix (Bio-Rad, Mississauga, Canada).

Transient transfection: Transient transfection was performed using Lipofetamine 2000 (Invitrogen) following manufacturer’s suggested procedures. Expression constructs for c-myc tagged full-length Nodal and constitutively active ALK7 (ALK7-ca) generated by our lab have been reported (Xu et al., 2004). Constitutively active ALK5 and ALK4 were kindly provided by Drs. Liliana Attisano and Jeff Wrana (University of Toronto, Canada) (Attisano et al., 1996; Wieser et al., 1995). pCS2-GFP was obtained from Dr. Cunming Duan (University of Michigan, MI, USA) (Li et al., 2005) while N53(encoding full length Nodal in pCS2) and N56 (encoding mature Nodal in pCS2) were generous gifts from Dr. Karuna Sampath (National University of Singapore, Singapore) (Tian et al., 2008). Wild type p27 (p27-wt) was a gift from Dr. Michele Pagano (New York University Cancer Institute, NY, USA) and two S10 mutants, p27-S10A and p27-S10D were provided by Dr. Michelle Olive (National Institute of Health, DC, USA) (Boehm et al., 2002). A p27 promoter construct (p27PF) which contains 3550 bp of the p27 promoter sequence upstream of the firefly luciferase gene was kindly provided by Dr. Toshiyuki Sakai (Kyoto Prefectual University of Medicine, Japan) (Inoue et al., 1999).
**Proliferation assays:** Cells proliferation was determined by either manual counting of live cells using Trypan blue staining or by WST-1 (4-(3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1, 3-benzene disulfonate) assay. For WST-1 assay, transiently transfected cells were seeded in a 96-well and cultured for 48 hours. Subsequently, 10 µl/well of WST-1 reagent (BioVision, CA) was added to the plate. After incubation at 37 °C for 1 hour, the absorbance was measured at 450 nm.

**Protein extraction and immunoblotting:** Cell lysates were prepared in radioimmune precipitation assay buffer (RIPA: 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 1% SDS). Equal amount of protein was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA). After blocking with 5% milk in TBS-T, the membrane was incubated with a respective primary antibody at 4 °C overnight. The membranes were subsequently probed with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. Signals were detected using ECL-Plus Kit (Amersham). Antibodies used for immunoblotting were p27^KIP1 (BD, 1:2000), phosphor-p27-T187 (Zymed, 1:200 and Santa Cruz, 1:500), phosphor-p27-T198 (Santa Cruz, 1:500), phosphor-p27-S10 (Zymed, 1:100 and Santa Cruz, 1:500), phosphor-p27-T157 (Santa Cruz, 1:500), cdk2 (Sigma, 1:1,000 and Santa Cruz, 1:500), cdk5 (Santa Cruz, 1:500), HA (Cell Signalling, 1:500), β-actin (Sigma, 1:10,000) and acetylated α-tubulin (Sigma, 1:200), stathmin (Santa Cruz, 1:500), phosphor-stathmin-S25 (Santa Cruz, 1:500), phosphor-stathmin-S38 (Santa Cruz, 1:500), Lamin B (Santa Cruz, 1:1,000), GAPDH (Santa Cruz, 1:2,000), RhoA (Santa Cruz, 1:500), Cyclin A (Santa Cruz, 1:1,000), Cyclin E (Santa Cruz, 1:1,000), rabbit, mouse and goat normal IgG (Santa Cruz, 1:5,000-10,000). Cell fractionation was performed using the NE-PER, Nuclear and Cytoplasmic Extraction kit (Pierce, USA).

**Immunofluorescence analysis:** Cells were rinsed with cold PBS, fixed with ice cold Methanol/Acetone in 1:1 ratio, and then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After blocking with 0.1% BSA in PBS for 30 min to 1 h, cells were incubated with primary antibodies (1:100 in blocking solution) overnight at 4 °C. Subsequently, cells were washed three times and incubated with fluorochrome-labeled secondary antibodies, Alexa Fluor 594 and 488 (Molecular, Eugene, OR, 1:300 in blocking milk), for 30 min. Finally, cells were
counterstained with 1 µg/ml of DAPI. Fluorescent microscopy was performed using Nikon Eclipse TE2000-U microscope (Melville, NY) at various magnifications.

**Co-Immunoprecipitation:** Cells were washed twice with ice-cold PBS and lysed with RIPA buffer containing 1 mM dithiothreitol, 1 mM Na$_3$VO$_4$, 5 mM NaF, 100 mM EDTA, 10 mg/ml aprotinin, and 100 mM phenylmethylsulfonyl fluoride. Protein samples (500-1000 µg) were incubated with 2-4 µg of antibody overnight at 4°C and then applied to pre-washed Agrose beads, conjugated with either Protein A or protein G for 2 h at 4°C. Agarose beads were then washed three times with RIPA buffer and immunoprecipitates were collected by boiling beads in 40-50 µl of 2x Laemmli buffer for 15 min. Finally, the supernatant was subjected to SDS-PAGE and Western blot analysis.

**Migration and invasion assays:** Migration and invasion assays on transwell inserts were performed as previously described (Nadeem et al., 2011). Briefly, migration assays were carried out using 5 µm polycarbonate membrane transwell inserts (Costar, Corning Inc. NY). Transfected cells were collected using “Accutase” (Innovative Technologies Inc. USA) and seeded on the top of the transwell insert at a density of 20,000 per filter. Cells were incubated with 1% FBS containing RPMI-1640 while 10% FBS containing RPMI-1640 was added outside the transwells to serve as a chemotactic agent. Twenty four hours post plating, cells were fixed and stained with Harleco Hemacolor Staining Kit (EMD, NJ). Non-migrated cells on the top of the membranes were wiped off using a cotton swab and membranes were liberated with a scalpel and mounted on slides for quantification. Transwell invasion assays were performed in the same fashion with the exception that transwell inserts were precoated with growth-factor–reduced Matrigel (BD Biosciences). Migrated or invaded cells were counted at 100 x magnification under the microscope. Invasion or migration index was calculated as the fold of invaded or migrated cells in the treated groups over the control group. Data was pooled from multiple experiments.

**Wound healing assays:** Wound scratch assays were performed as described earlier (Nadeem et al, 2011). Briefly, cells were grown to confluence in 6-well culture plates. A wound was then created across the well down the middle. Cells were washed and treated with 20µM tubacin (ChemicTek, Indianapolis, IN, USA) or its vehicle in reduced serum medium. Pictures were
taken at 0 and 24 h after treatment.

**Statistical analysis:** Data are presented as mean±SEM. Differences among several groups were determined by one-way analysis of variance, followed by Student-Newman-Keul's test using GraphPad InStat software. For comparison between two groups, Student's $t$ test was used. $P < 0.05$ was considered significant. All experiments have been done at least three times.
Acknowledgment:
This study was supported by a grant from Canadian Institute of Health Research (MOP-81370) to CP. LN was supported by an Ontario Graduate Scholarship and CP was a recipient of a Mid-Career Award from OWHC/CIHR.
References


Legends to figures:

Fig.1. **Nodal increases p27 level via multiple mechanisms:** A) Nodal upregulates p27 mRNA. Cells were treated with recombinant Nodal (rNodal, 500 ng/ml) for 24 h. Total RNA was extracted, reversed transcribed and p27 mRNA levels were assessed using qPCR (n=4 replicates in a representative experiment). B) Nodal enhances p27 mRNA stability. Cells were serum starved for 24 h, serum stimulated for 4 h and then treated with 5 μM Actinomycin D with control medium (C) or 500 ng/ml rNodal (rN). Total RNA was extracted at every 20 min post treatment, p27 and GAPDH levels were determined by RT-PCR and quantified through densitometric analysis (n=3 experiments). C) Transfection of Nodal (N) or constitutively active ALK7 (ALK7-ca, CA) resulted in an increase in p27 mRNA levels when compared to the empty vector (EV) transfected cells (n=3 experiments). D) Nodal increases p27 protein stability. Cells were serum starved for 24 h, serum stimulated for 4 h and then treated with 5μg/ml cycloheximide with or without rNodal. Cell lysates were collected at the indicated time points and subjected to Western Blot analyses. E) Nodal inhibits p27 phosphorylation at T187. Treatment with rNodal for 12h resulted in a decrease in phosphor-p27 T187 levels. ***, p<0.0001; **, p<0.001; *, p<0.05.

Fig.2. **Nodal induces p27 and cdk2 nuclear export.** A) Immunofluorescent staining of p27 and cdk2 in cells transfected with empty vector (EV), N53 (encodes full length Nodal), or N56 (encodes the mature Nodal). Double immunostaining was performed 48 h after transfection, using anti-p27 and anti-cdk2 antibodies. B) Effects of Nodal, TGF-β, and Activin on p27 and cdk2 subcellular localization. Cells were synchronized by serum starvation for 24 h and then treated with 500ng/ml rNodal, 10ng/ml TGF-β1, or 100ng/ml activin-A. Double immunostaining was performed 48 h post treatment using anti-p27 and anti-cdk2 antibodies. C) Cells were transfected with constitutively active receptors, ALK4-ca, ALK5-ca and ALK7-ca and immunostained for p27 and cdk2 at 48 h after transfection. Scale bars = 50µm.

Fig.3. **Phosphorylation of p27 at S10 is critical for Nodal-induced p27 nuclear export.** A) Nodal induces p27 phosphorylation at S10. Cells were serum starved for 24 h and then treated with 500 ng/ml rNodal (rN). Nuclear and cytoplasmic fractions were prepared at 6 and 16 h after
treatment and subjected to immunoblotting probed by anti-phosphor-p27-S10 antibody. Lamin B and GAPDH were used as fractionation and loading controls. B) Nodal enhanced p27 phosphorylation at S10 but decreased p27 phosphorylation at T157, T198, and T187. Cells were treated with or without Nodal for 24 h and cytoplasmic (Cyt) and nuclear (Nuc) fractionation was performed. C) Cells were transfected with HA-tagged wild type p27 (p27-WT), or p27-S10 mutants, p27-S10D or p27-S10A, and co-transfected with Nodal (N56) or control (EV) plasmids. Double Immunostaining for anti-HA and Anti-p27 was performed at 48 h after transfection. Scale bar = 50 µm.

Fig.4. p27 mediates the effect of Nodal on cell proliferation. A) Validation of siRNA mediated knockdown of p27 and cdk2. Cells were transfected with 100 nM control, p27 siRNA, or cdk2 siRNA. Cell lysates were subjected to immunoblotting using anti-p27 and anti-cdk2 antibodies. B) Assessment of cell proliferation by manual cell counting. Cells were transfected with control, cdk2 siRNA, or p27 siRNA and treated with or without rNodal for 48 h. Data represents mean ± SEM (n=3 experiments). Different letters denote statistical significance. C) WST-1 Assay to determine cell proliferation/viability. Cells were transfected with control, cdk2 siRNA, or p27 siRNAs, together with Nodal expressing plasmid or its empty vector control (EV). WST reagent was added at 48 h after transfection and absorbance was read at 450 nm. Data represents mean±SEM (n=3 experiments). Different letter denote statistical significance. D) Representative pictures of HTR-8/SVneo cells transfected with control, cdk2 siRNA, or p27 siRNA and treated with control or rNodal for 48 h are shown. Scale bar = 100 µm.

Fig.5. p27 mediates the effect of Nodal on cell migration and invasion. A) Transwell migration assay was performed by seeding equal number of cells transfected with control siRNA or p27 siRNA on transwell inserts and then treated with or without rNodal (500ng/ml) for 24 h. Data represent mean ± SEM (n=3 experiments). B) Transwell matrigel invasion assay was performed by seeding equal number of transfected cells (with control or p27 siRNAs) on matrigel-coated filters and treated with or without 500 ng/ml rNodal. Cells were fixed and stained at 24 h post seeding and invaded cells were counted. Data represent mean ± SEM (n=3 experiments). Representative pictures are shown. Scale bar = 1mm.
**Fig. 6.** Nodal enhances the interaction between p27/cdk2/cdk5 and stathmin and induces stathmin phosphorylation. A) Nodal enhanced the association of p27 with cdk2, cyclin E, cdk5 and stathmin but inhibited the binding of p27 to RhoA and cyclin A. Control (C) or rNodal (rN)-treated cell lysates were subjected to immunoprecipitation by anti-p27 antibody, followed by immunoblotting using the indicated antibodies. Cell lysate (input) was also subjected to immunoblot analyses. B) Cells were treated with control (C) or rNodal (rN) and cell lysates were subjected to immunoprecipitation by anti-stathmin antibody, followed by immunoblotting with cdk2, cdk5 p27 and stathmin. C) Nodal induces stathmin phosphorylation. Cells were treated with vehicle, 10µM cdk2 inhibitor (cdk2 Inh), or cdk2/5 inhibitor, along with control (C) or rNodal (rN) for 24 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. Nodal induced phosphorylation of stathmin at S25 and S38 and this effect was reduced by cdk2/5 inhibitor.

**Fig. 7.** Mechanisms by which p27 mediates the anti-migratory effect of Nodal. A) Nodal enhances α–tubulin acetylation/stability. Immunostaining of acetylated α–tubulin in empty vector (EV) or Nodal-transfected cells. Pictures were taken at constant exposure time. B) An HDAC6 inhibitor, tubacin, enhanced α–tubulin acetylation. Immunostaining of acetylated α–tubulin in vehicle- or tubacin-treated cells (20µM for 24 h). Pictures were taken at constant exposure time. C) Increased acetylation of α–tubulin inhibits cell migration. Wound scratch assays were performed using cells treated with 20µM tubacin or its vehicle for 24 h. Representative pictures is shown. D) Proposed model for mechanism of Nodal regulated inhibition of trophoblast cell migration. Nodal induces p27 and cdk2 nuclear translocation and enhances the interaction of cytosolic p27, cdk2, cdk5 and stathmin. Within the complex stathmin becomes hyperphosphorylated and loses its tubulin depolymerisation function resulting in tubulin stabilization and inhibition of cell motility. Scale bars = 50 µm.
A  

**Relative p27 mRNA Level**

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E  

**Western Blot Images**

- Control: p27-T187, p27, β-actin
- rNodal: p27-T187, p27, β-actin
Nadeem et al., Fig. 3

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- p27-S10
- p27
- Lamin B
- GAPDH

Cytoplasmic  Nuclear  Cytoplasmic  Nuclear

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- p27-S10
- p27-T157
- p27-T198
- p27-T187
- p27
- Lamin B
- GAPDH

C

EV

- Anti-p27
- Anti-HA

Nodal

- p27-WT
- p27-S10A
- P27-S10D

- Anti-p27
- Anti-HA
Nadeem et al., Fig. 4

A

Control  p27 siRNAs
β-actin  p27

Control  cdk2 siRNAs
β-actin  cdk2

B

Control  cdk2  p27 siRNAs

Relative Cell Number

C

Control  cdk2  p27 siRNAs

Absorbance

D

Control  cdk2  p27 siRNAs

Control  rNodal
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C  

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Nadeem et al., Fig. 7

**A**
α-acet tubulin | DAPI
---|---
EV | Nodal

**B**
α-acet tubulin | DAPI
---|---
DMSO | Tubacin

**C**
DMSO | Tubacin
---|---
0h | 24h

**D**
\[ \text{Nodal} \downarrow \]
\[ \uparrow \text{cytoplasmic p27 and cdk2} \]
\[ \downarrow \]
\[ \uparrow \text{p27/cdk2/cdk5/stathmin} \]
\[ \downarrow \]
\[ \uparrow \text{pStathmin} \]
\[ \downarrow \]
\[ \text{Tubulin stabilization} \]
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\[ \text{Migration/invasion} \]