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Secreted phosphoprotein 1 binds integrins to initiate multiple cell signaling pathways, including FRAP1/mTOR, to support attachment and force-generated migration of trophectoderm cells

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ABSTRACT

Attachment and migration of trophectoderm (Tr) cells, hallmarks of blastocyst implantation in mammals, are unique uterine events. Secreted phosphoprotein 1 (SPP1) in the uterus binds integrins on conceptus Tr and uterine luminal epithelium (LE), affecting cell–cell and cell–matrix interactions. The signal transduction pathways activated by SPP1 and integrins in conceptuses have not been elucidated. Results of this study demonstrate that SPP1 binds αvβ3 and α5β1 integrins to induce focal adhesion assembly, a prerequisite for adhesion and migration of Tr, through activation of: 1) P70S6K via crosstalk between FRAP1/mTOR and MAPK pathways; 2) mTOR, PI3K, MAPK3/MAPK1 (Erk1/2) and MAPK14 (p38) signaling to stimulate Tr cell migration; and 3) focal adhesion assembly and myosin II motor activity to induce migration of Tr cells. These cell signaling pathways, acting in concert, mediate adhesion, migration and cytoskeletal remodeling of Tr cells essential for expansion and elongation of conceptuses and attachment to uterine LE for implantation.

1. Introduction

The mammalian target of rapamycin (mTOR/FRAP1) cell signaling pathway is an evolutionarily conserved serine/threonine kinase located downstream of phosphatidylinositol 3-kinase that controls cell growth and proliferation through regulation of protein synthesis (Hay and Sonenberg, 2004; Wullschleger et al., 2006), as well as initiation of mRNA translation, ribosome synthesis, expression of metabolism-related genes, autophagy and cytoskeletal reorganization (Kim et al., 2002). mTOR is a "nutrient sensing system" that may be stimulated by molecules including insulin-like growth factor 2 (IGF2) and selected amino acids (Nielsen et al., 1995; Kimball et al., 1999; Martin and Sutherland, 2001) to support blastocyst/conceptus (embryo and extra-embryonic membranes) development. Homozygous *Frap1* null mice die shortly after implantation due to impaired cell proliferation and hypertrophy in both the embryonic disc and trophoblast (Murakami et al., 2004).

Cell attachment and migration are key events in implantation of blastocysts in uteri of all mammals. Trophectoderm cells (Tr) of blastocysts attach to the uterine luminal epithelium (LE) for juxtaposition of conceptus and maternal circulations, leading to the establishment of a functional placenta. Attachment of Tr to LE is facilitated by a mosaic of interactions between integrins and extracellular matrix (ECM) proteins which contribute to stable adhesion at implantation (Burghardt et al., 2002; Lessey and Castelbaum, 2002; Aplin and Kimber, 2004). In sheep, conceptus elongation occurs prior to and is a prerequisite for the initiation of implantation and involves a rapid transition from spherical to tubular and filamentous forms between Days 10 and 15. Elongation of conceptuses involves extensive cytoskeletal reorganization in Tr cells to support their hypertrophy and migration (Geisert and Yelich, 1997). Implantation in sheep is noninvasive with increasing apposition and adhesion between Tr and uterine LE between Days 18 and 50 to 60 of gestation (Guillomot, 1995).

Secreted phosphoprotein 1 (SPP1/osteopontin), a multifunctional ECM protein, binds to cell surface integrin receptors to regulate basic cell–cell and cell–matrix interactions essential to cell adhesion, migration and proliferation (Sodek et al., 2000; Johnson et al., 2003). SPP1 contributes to implantation in several mammalian species (Johnson et al., 2003) and multiple integrin receptors for SPP1 are present on Tr and LE of humans and domestic animals, some of which

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increase during the peri-implantation period (Lessey, 1994; Burghardt et al., 2002; Johnson et al., 2003). Binding of SPP1 to integrins results in activation of integrin receptors and recruitment of cytoskeletal proteins to form focal adhesions in Tr cells (Erikson et al., 2009), and *Spp1*$^{-/-}$ and *Spp1*$^{+/-}$ mice display decreased pregnancy rates at mid-gestation (Weintraub et al., 2004).

SPP1 induces motility in human trophoblast cells through mTOR signaling (Al-Shami et al., 2005) and rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins stimulated by insulin-like growth factor (IGF1) such as focal adhesion kinase (FAK) (Liu et al., 2008). These results suggest a role for SPP1-induced mTOR complex signaling in key events of pregnancy. However, little is known about downstream transcription factor activation and transcription factor-mediated gene expression induced through SPP1-integrin interactions in mammalian Tr cells. Therefore, this study was designed to identify relationships and crosstalk between multiple membrane and intracellular cell signaling cascades activated by SPP1, including mTOR, and integrin binding to ovine Tr cells, that control cell proliferation, migration, attachment and adhesion in conceptuses during the peri-implantation period of pregnancy. Results of these studies provide strong evidence for SPP1-induced integrin-mediated force-generated migration and cell adhesion mechanisms in Tr cells utilizing mTOR, MAPK14 (p38) and MAPK1/MAPK3 (Erk1/2), as well as a novel link between p38 and mTOR signaling pathways.

2. Results

2.1. SPP1 binds αvβ3 and α5β1 integrin heterodimers to activate focal adhesion assembly at the basal surface of trophectoderm cells

Immunoprecipitations of integrins in oTr1 cell lysates were performed using antibodies directed to integrin subunits reported to be expressed on ovine Tr cells including α4, α5, αv, β1, β3, αvβ3 and αvβ5 (Fig. 1A, first panel). Affinity chromatography was used to evaluate binding of integrins on oTr1 cells to SPP1. Detergent extracts of surface-biotinylated oTr1 cells were incubated with bovine milk (bSPP1)-Sepharose in the presence of 1.5 mM Mg^{2+} and 1.5 mM Mn^{2+}. After incubation, the column was washed in the presence of 1.5 mM Mg^{2+} and 1.5 mM $Mn^{2+}$and eluted with EDTA to chelate cations and release bound integrins. Integrins eluted in fractions E2, E3 and E4, with α-integrin chains migrating at approximately 170 kDa and β-integrin chains migrating at 100–120 kDa (Fig. 1A, second panel).

Successful immunoprecipitation of labeled oTr1 integrins occurred with antibodies to αv, β3 and α5 integrin subunits, as well as an antibody to the integrin αvβ3 heterodimer (Fig. 1A, third panel; note that the bands detected migrated at the same sizes as the bands in the eluates shown in the second panel). An antibody to the αv integrin subunit also precipitated a β chain, presumed to be the β3 integrin subunit, as an antibody to the β3 integrin subunit precipitated an α chain at the same relative size as the bands precipitated by an antibody to the αvβ3 heterodimer. Thus, the αvβ3 integrin on oTr1 cells binds SPP1.

We were unable to immunoprecipitate the α5 or β1 integrin subunits from membrane extracts of biotinylated oTr1 cells that were eluted from an SPP1-Sepharose column or identify α5 or β1 integrin subunits from the same eluates by Western blotting (Fig. 1A, fourth panel). However, based upon the α5 integrin subunit immunoprecipitation results from labeled oTr1 cells, the fact that the β1 subunit is the only known binding partner for α5, as well as the complexity of α5β1 integrin binding to ECM, immunofluorescence studies were performed to evaluate the impact of functional binding of αvβ3 and α5β1 integrins at the basal surface of oTr1 cells.

Because β1 integrins, but not the αvβ3 heterodimer, bind collagen (Humphries et al., 2006), slides were coated with bSPP1 or collagen Type I and immunostained using antisera to αvβ3, α5, β1 or talin (TLN). The αvβ3 integrin heterodimer and TLN were detected as large aggregates at the basal surface of oTr1 cells on slides coated with bSPP1 (Fig. 1B). The β1 integrin subunit was also detected in small aggregates at the basal surface of oTr1 cells on slides coated with bSPP1 and larger β1 integrin aggregates were detected in cells grown on collagen (Fig. 1C). These results suggest that the αvβ3 and possibly α5β1 integrins functionally aggregate at the basal surface of oTr1 cells in response to bSPP1 to elicit focal adhesion assembly and integrin activation during ovine Tr cell attachment to immobilized bSPP1.

The α5 and β1 aggregates on the basal surface of cells grown on slides coated with SPP1 are intriguing and may provide insight into the function and binding of this integrin in Tr during pregnancy. Unlike αvβ3 which binds to the RGD sequence in numerous ECM proteins, α5β1 integrin binding to ECM appears to be restricted to fibronectin (FN) and secreted phosphoprotein 1 (SPP1) (Humphries et al., 2006). Further, the binding of both SPP1 and FN to α5β1 is more complex than the interaction between SPP1 and other integrin receptors. For instance, adhesion of SPP1 to the αvβ3 integrin is not affected by alteration of SPP1 structure at, or adjacent to, the integrin recognition sequence. In contrast, binding of SPP1 and FN to α5β1 requires not only binding to the RGD portion of the integrin recognition sequence, but also binding to both a synergy site (the PHSRN sequence in the FN III_9 repeat for FN or the SVVYGLR site for SPP1) and a heparin binding site (Yokosaki et al., 2005). Both FN and SPP1 have a heparin binding site which, in FN, is required for focal adhesion formation (Okina et al., 2009). The heparin binding site in SPP1 overlaps with the thrombin cleavage site and likely represses SPP1 proteolysis (Kon et al., 2008). It is also noteworthy that the N-terminal fragment generated by thrombin cleavage containing both the RGD and SVVYGLR sequences is a stronger ligand for the α5β1 integrin than full length SPP1 (Yokosaki et al., 2005). Therefore, for optimal SPP1 binding to α5β1, the proper presentation of the protein is required, and this presentation is likely different when the protein is coated on the rigid surface of a glass slide than when it is linked to a Sepharose bead. It is possible that the α5β1 integrin isolated in a membrane extract will not bind efficiently to SPP1 without the availability of a synergy site and a heparin binding site. It is also likely that the SPP1 on beads remains in the intact full-length form, whereas it is quite conceivable that SPP1 at the base of cultured cells is converted into its thrombin cleavage fragment by serine proteases secreted by the oTr1 cells themselves. Interestingly, cleavage of SPP1 occurs *in vivo* within the lumen of pregnant animals; the source of enzymes responsible for this process may be elongating conceptus trophectoderm (Johnson et al., 1999; Garlow et al., 2002). We hypothesize that our immunofluorescence data demonstrating aggregation of α5 and β1 integrin subunits into focal adhesions at the basal surface of oTr1 cells on SPP1-coated glass contain the unique complement of binding sites to utilize the α5β1 integrin to bind to SPP1 in contrast to the α5β1 in membrane extracts attached to Sepharose beads. While we cannot definitively state that SPP1 binds α5β1 integrin on oTr1 cells due to the chromatography results, we are reticent to exclude this possibility due to the focal adhesion assembly at the base of oTr1 cells in culture. Indeed, the more physiological configuration of the SPP1 in the latter case suggests that SPP1 likely binds the α5β1 integrin on oTr1 cells.

To establish the ability of ovine Tr cells to undergo integrin activation and assemble focal adhesions *in vivo* during implantation, cryosections of Day 20 ovine implantation sites containing conceptus Tr cells attached to uterine LE cells were also subjected to immunofluorescence analyses. Alpha-actinin (ACTN) was detected in aggregates within conceptus Tr at implantation sites (Fig. 1D). ACTN is an actin binding and bundling protein associated with the cytoplasmic tail of beta integrin subunits in focal adhesions, as well as the zonula adherens (adherens junctions). ACTN became increasingly distributed across the apical surfaces of uterine LE and conceptus Tr cells on Day 20 of pregnancy, whereas no changes in ACTN distribution were associated with adherens junctions in adjacent uterine glandular epithelia within or outside implantation sites. This indicates recruitment of ACTN to the apical surface of uterine LE and conceptus Tr during assembly of focal adhesions.