SUMMARY

ANGIOGENESIS AND MALIGNANT TRANSFORMATION: ROLE AND MECHANISM OF ACTION OF ACTIVIN A.

DOCTORATE THESIS BY
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Angiogenesis, the generation of new vessels from pre-existing ones, is an indispensable prerequisite for the progression and metastasis of solid tumors. Tumor angiogenesis appears to be regulated either by hypoxia or by mutations of oncogenes and tumor suppressor genes. We have addressed activation of angiogenesis in neuroblastoma, a malignancy characterised by the near-exclusive amplification and overexpression of the N-*myc* oncogene. Amplification of N-*myc* oncogene is a frequent event in advanced stages of human neuroblastomas and correlates with poor prognosis and enhanced vascularization.

In our previous studies, activin A was identified as an N-myc-regulated molecule, which can regulate endothelial cell proliferation. Activin A belongs to the TGF-β superfamily of secreted polypeptide growth factors that play critical roles during embryogenesis and in maintaining tissue homeostasis in adult life. The TGF-β/activin family members signal through heteromeric complexes of transmembrane type I and type II serine-threonine kinase receptors. The type II receptor kinase phosphorylates the type I receptor kinase which in turn phosphorylates the downstream transducer proteins Smad2 and Smad3. The latter associates with Smad4 and the resulting complex translocates to the nucleus, where they control the transcription of target genes. The Smad-binding protein SARA recruits Smad2 and Smad3 for phosphorylation by the type I receptors.

In the present study we show that activin A inhibits the proliferation of neuroblastoma cells and that activin A-expressing xenograft tumors in mice exhibit reduced neovascularization and suppressed growth. Moreover, we show that activin A inhibits endothelial cell responses important for angiogenesis, such as degradation of

extracellular matrix, migration and proliferation. Recombinant adenoviruses expressing activin A and the constitutively active type I receptor (ALK4) can inhibit proliferation induced by the angiogenic factors FGF-2 and VEGF, mimicking the effects of exogenously introduced activin A. Activin A inhibits endothelial cell proliferation not only by increasing transcriptional activity from specific CdkI promoters like p15, p21 and p27, but also by inhibiting the VEGF-induced mitogenic pathway ERK1/2 via down regulation of VEGFR-2 expression.

We have also explored in more detail the activin A signaling in endothelial cells, and we have found a critical role for the early endocytic compartment in regulating Smad-dependent signaling. Not only is SARA localized to on early endosomes, but also its minimal FYVE finger sequence is sufficient for early endosomal targeting. Expression of a SARA mutant protein lacking the FYVE finger inhibits downstream activin A signaling in endothelial cells. Moreover, a dominant-negative mutant of Rab5, a crucial protein for early endosome dynamics, causes phosphorylation and nuclear translocation of Smads leading to constitutive transcriptional activation of a Smad-dependent promoter in endothelial cells. As inhibition of endocytosis using the K44A negative mutant of dynamin and RN-tre did not lead to activation of Smad-dependent transcription, the effects of the dominant-negative Rab5 are likely to be a consequence of altered membrane trafficking of constitutively formed TGF-β/activin type I/II receptor complexes at the level of early endosomes.

Beyond the role of early endosomes in activation of Smads, the latter do not appear to mediate exclusively all actions of activin A. Indeed, whereas constitutively active forms of Smad2 and Smad3 inhibited endothelial cell proliferation, dominant negative forms of Smad3 and Smad4 reversed this inhibition by only 20-30%. In conjunction with the observation that activin A reduced activation of $p70^{S6K}$ kinase, an important protein for progression through G_1, these data indicate that in addition to activation of Smads, deactivation of $p70^{S6K}$ kinase contributes to the antimitotic effects of activin A in endothelial cells.

Collectively, we have showed that activin A plays an important role in malignant transformation, we have documented its antiangiogenic action, and we have found novel aspects of activin A signaling in endothelial cells.

1: Panopoulou E, Murphy C, Rasmussen H, Bagli E, Rofstad EK, Fotsis T. Related Articles, Links

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The tumor suppressor function of activin A, together with our findings that activin A is an inhibitor of angiogenesis, which is down-regulated by the N-MYC oncogene, prompted us to investigate in more detail its role in the malignant transformation process of neuroblastomas. Indeed, neuroblastoma cells with restored activin A expression exhibited a diminished proliferation rate and formed smaller xenograft tumors with reduced vascularity, whereas lung metastasis rate remained unchanged. In agreement with the decreased vascularity of the xenograft tumors, activin A inhibited several crucial angiogenic responses of cultured endothelial cells, such as proteolytic activity, migration, and proliferation. Endothelial cell proliferation, activin A, or its constitutively active activin receptor-like kinase 4 receptor (ALK4T206D), increased the expression of CDKN1A (p21), CDKN2B (p15), and CDKN1B (p27) CDK inhibitors and down-regulated the expression of vascular endothelial growth factor receptor-2, the receptor of a key angiogenic factor in cancer. The constitutively active forms of SMAD2 and SMAD3 were both capable of inhibiting endothelial cell proliferation, whereas the dominant-negative forms of SMAD3 and SMAD4 released the inhibitory effect of activin A on endothelial cell proliferation by only 20%. Thus, the effects of activin A on endothelial cell proliferation seem to be conveyed via the ALK4/SMAD2-SMAD3 pathways, however, non-SMAD cascades may also contribute. These results provide novel information regarding the role of activin A in the malignant transformation process of neuroblastomas and the molecular mechanisms involved in regulating angiogenesis thereof.

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Transforming growth factor beta (TGFbeta) receptors require SARA for phosphorylation of the downstream transducing Smad proteins. SARA, a FYVE finger protein, binds to membrane lipids suggesting that activated receptors may interact with downstream signaling molecules at discrete endocytic locations. In the present study, we reveal a critical role for the early endocytic compartment in regulating Smad-dependent signaling. Not only is SARA localized on early endosomes, but also its minimal FYVE finger sequence is sufficient for early endosomal targeting. Expression of a SARA mutant protein lacking the FYVE finger inhibits downstream activin A signaling in endothelial cells. Moreover, a dominant-negative mutant of Rab5, a crucial protein for early endosome dynamics, causes phosphorylation and nuclear translocation of Smads leading to constitutive (i.e. ligand independent) transcriptional activation of a Smad-dependent promoter in endothelial cells. As inhibition of endocytosis using the K44A negative mutant of dynamin and RN-tre did not lead to activation of Smad-dependent transcription, the effects of the dominant-negative Rab5 are likely to be a consequence of altered membrane trafficking of constitutively formed TGFbeta/activin type I/II receptor complexes at the level of early endosomes. The results suggest an important interconnection between early endosomal dynamics and TGFbeta/activin signal transduction pathways.

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