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ISREC

Watch thy neighbor: Proprotein convertases activate TGFß-related signals across tissue boundaries

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Daniel Constam obtained his doctoral degree in natural sciences at the Swiss Federal Institute of Technology (ETH), Zürich in 1993. After postdoctoral work with E. J. Robertson at Harvard University, Cambridge MA, he joined ISREC as an associate scientist in 2000.

Embryonic development of epithelial tissues, and their continuous regeneration from pools of self-renewing stem cells in the adult is dependant on reciprocal inductive interactions with surrounding stromal tissues. Disturbance of this molecular cross-talk between neighboring cells by somatic mutations, inflammation or other epigenetic regulation of gene expression is thought to play a key role in the process of carcinogenesis and the transformation of benign tumors into malignant, invasive carcinomas. Therefore, one possible avenue to improve existing cancer therapies might be to design new strategies that restore normal communication among the tissues involved. Although, to identify suitable drug targets within these regulatory signaling networks, it will be necessary to define the hierarchy among individual components, and to determine their relevant functions in the homeostasis of healthy versus cancerous tissues. As a model system, my lab studies the inductive tissue interactions that control the differentiation of pluripotent stem cell populations in the early post-implantation stage mouse embryo. Here, I summarize some of our recent findings how the first distinct cell lineages recognized in the mammalian fetus communicate with each other to coordinate multiple cell fate decisions during the process of gastrulation.

Axis formation in the mammalian embryo

During the initial stages of development, mammalian embryos must generate several extraembryonic tissues that are essential for survival in the uterus, but which do not themselves contribute to the resulting animals. As a result, the conceptus is already a complex, asymmetric structure long before the embryo itself begins to acquire obvious pattern. Thus, shortly before implantation into the uterus, the process of blastulation leads to the segregation of the inner cell mass (ICM) from the surrounding trophectoderm, a squamous epithelium destined to generate the trophoblast and the extraembryonic ectoderm (**Fig. 1**). Within the following 24 hours, cells on the surface of the ICM facing the blastocoelic cavity differentiate to become primitive endoderm, eventually giving rise to the extraembryonic parietal and visceral endoderm. The remainder of the ICM proliferates, and by day E5.5 undergoes a process of cavitation to form the epiblast, which is the sole founder tissue of the entire embryo. This cylindrical structure is eventually converted during gastrulation into the three primary germ layers, namely ectoderm,

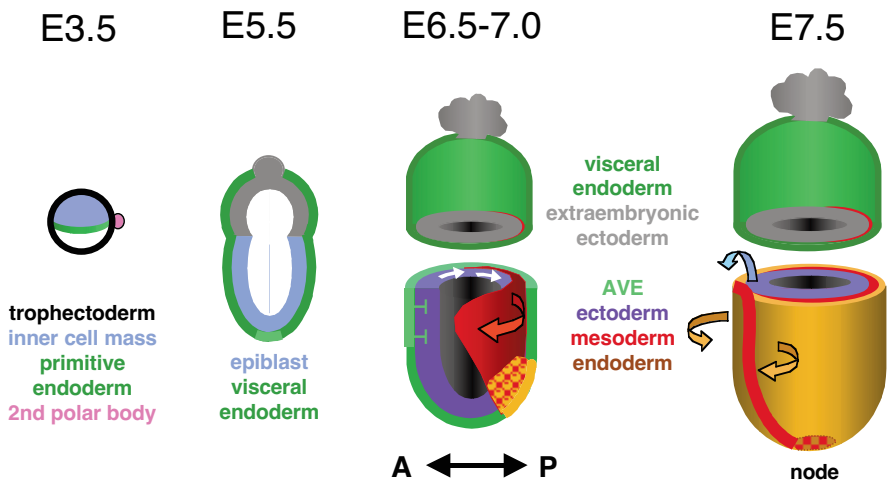

Fig. 1. *Axis specification and germ layer formation in the gastrulating mouse embryo. Between embryonic day E3.5 and E6.0, expansion of polar trophectoderm and the inner cell mass gives rise to extraembryonic ectoderm (grey) and the epiblast (blue), respectively. Primitive endoderm at the distal tip differentiates and is displaced to the prospective anterior side to become anterior visceral endoderm (AVE, bright green), and to inhibit posterior cell fates in adjacent epiblast. At the opposite pole, epiblast cells ingress into the primitive streak (white arrows) to give rise to mesoderm and the definitive endoderm germ layer. Towards late gastrulation (E7.5), anterior mesoderm will form the node and the notochord (red line), whereas definitive endoderm displaces the visceral endoderm to the extraembryonic region, and eventually folds up into the primitive gut tube (brown arrows) underneath the headfold (blue arrow).*

mesoderm, and definitive endoderm. In this process, mesodermal and endodermal precursor cells within the epiblast ingress at the prospective posterior pole to form the so-termed primitive streak. Within the streak, they undergo an epithelial-mesenchymal transition, delaminate, and eventually migrate along the outer surface of the egg cylinder towards the anterior pole, thereby displacing the visceral endoderm to the extraembryonic region. The primitive streak, which anticipates the future rostral-caudal (or anteroposterior, A/P) body axis, appears to be positioned by the so-termed anterior visceral endoderm (AVE). This subpopulation of VE cells initially forms at the apex of the egg cylinder, and subsequently moves to the prospective anterior pole to inhibit posterior cell fates in the adjacent epiblast, thereby allowing the formation of anterior structures (Shawlot and Behringer, 1995; Thomas and Beddington, 1996; Rhinn et al., 1998; Perea-Gomez et al., 2002).

The signaling cascade that specifies the AVE is triggered by a secreted protein of the TGFß family termed Nodal (Lu et al., 2001). Interestingly, the same protein subsequently also induces mesodermal and endodermal cell fates and primitive streak formation in the epiblast. Thus, in the absence of a functional Nodal gene, embryos arrest at the egg cylinder stage lacking both anterior and posterior identity (Brennan et al., 2001). In the VE, Nodal induces transcription factors such as Otx2 and Lhx1, and expression of Cer-l and Lefty-1. The latter are secreted proteins that act as negative feedback inhibitors to antagonize Nodal signaling in prospective anterior cells (Piccolo et al., 1999; Perea-Gomez et al., 2002). By contrast in the epiblast, Nodal amplifies its own expression by autoregulation (Norris et al., 2002) and through positive feedback loops mediated by the induction of the Nodal co-receptor Cripto, and other down-stream effectors such as Wnt3, Fgf8, and Bmp4 (Brennan et al., 2001). Eventually, these feedback loops are thought to establish a graded Nodal signal that orchestrates cell movements and patterns all three germlayers, with peak levels inducing posterior, and low levels specifying anterior cell fates.

The secreted proprotein convertases Spc1 and -4 activate the Nodal precursor

Almost all TGFß family members, including Nodal, are initially synthesized as precursor proteins which must undergo endoproteolytic cleavage to remove an inhibitory pro peptide. We and others proposed that this cleavage may be mediated by multiple proteases of the subtilisin-like proprotein convertase (SPC) family thought to reside in the trans-Golgi network (Dubois et al., 1995; Constam et al., 1996; Constam and Robertson, 1999). Although, the only known murine Spc activities that we could detect before mid-gastrulation stages (E7.0) comprise Spc1 and Spc4, which we found to be transcribed specifically in the ExE. By comparison, Nodal is expressed in a complementary fashion in the epiblast and, at low levels, in the overlying visceral endoderm (**Fig. 2**). Therefore, we asked whether SPCs may process Nodal after secretion. Indeed, previous reports indicated that tissue culture cells release soluble forms of both SPC1 and SPC4 into the culture medium, which we confirmed to be true also for the murine homologs after transient transfection in COS1 cells (**Fig. 2, right panel**). When co-transfected together with Nodal, both Spc1 and –4 stimulated precursor cleavage, whereas Spc7, which is not secreted, was inactive (Constam and Robertson, 1999). However, no cleaved product was detected in cell lysates. Furthermore, soluble forms of Spc1 and Spc4 enhance Nodal cleavage also in the absence of cells. This cleavage is inhibited after ablation of the SPC recognition motif RXXR, and by the SPC inhibitor decanoyl-RVRR-chloromethylketone (Beck et al., 2002). Finally, we also observed significant Nodal processing if recombinant precursor was incubated with embryonic stem cells. Cleavage was inhibited, however, if both Spc1 and –4 were inactivated by homologous recombination. Transient transfection with an Spc1 expression vector partially restored Nodal cleavage, confirming that lack of processing was due to loss of Spc1/4 expression (Beck et al., 2002). Together, these results show that Spc1 and –4 are necessary and sufficient to cleave Nodal in tissue culture.

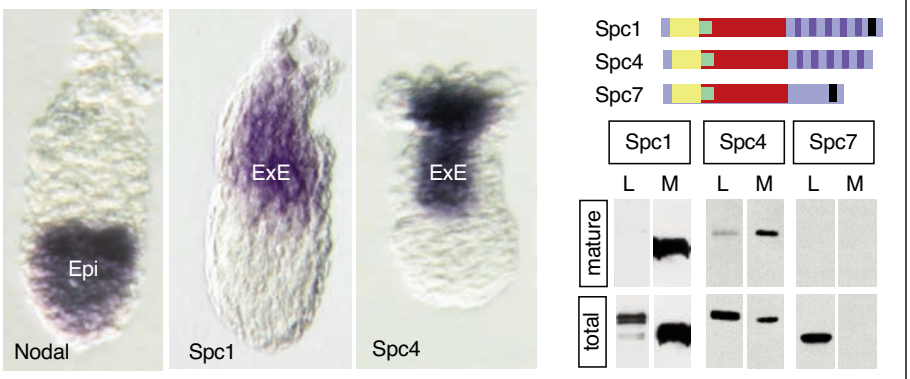

Fig. 2. *At the onset of gastrulation (E5.5), mRNAs encoding the serine proteases Spc1 and -4 are transcribed in the extraembryonic ectoderm (ExE) adjacent to their candidate substrate Nodal, which is produced in the epiblast (Epi) and overlying visceral endoderm. Spc1, -4 and -7 tagged with a Flag epitope (green box) accumulate in lysates (L) of transfected COS1 cells, although mainly in their inactive zymogenic form. The mature forms of Spc1 and Spc4 in which the pro peptide (yellow) was removed by autocleavage are released into the culture medium (M).*

Spc1 and –4 are required to activate the Nodal pathway in adjacent tissues

Single mutant embryos lacking Spc1 or -4 display a variety of defects, for example in cardiomyocyte specification and heart looping morphogenesis, and in the establishment of left-right

asymmetry of the visceral situs (Roebroek et al., 1998; Constam and Robertson, 2000). However, they both gastrulate, indicating that Nodal is sufficiently active to promote germ layer formation. Therefore, we asked whether Spc1 and –4 during early stages compensate for each other in activating Nodal in the embryo, as observed in ES cells. In keeping with this idea, we found that compound mutant embryos lacking both of these convertases arrest development at the egg cylinder stage and phenocopy almost all aspects of Nodal mutants (Beck et al., 2002). Thus, Nodal expression is significantly attenuated due to inhibition of autoinduction, and eventually fails to become posteriorized, which is attributed to the lack of a functional AVE (**Fig. 3**). Furthermore, in the absence of Spc1/4 activities, Nodal fails to induce its own co-receptor Cripto and a number of transcription factors implicated in maintaining pluripotent stem cells in the epiblast and ExE, including Pou5f1 and Eomesodermin. Also the expression of other downstream effectors such as Wnt3, Fgf8, and Bmp4 is severely inhibited, explaining why mesodermal and endodermal markers such as Brachyury (T) and Foxa2 are lost. These results show that normal Nodal signaling in the embryo is dependant on Spc1/4 activities.

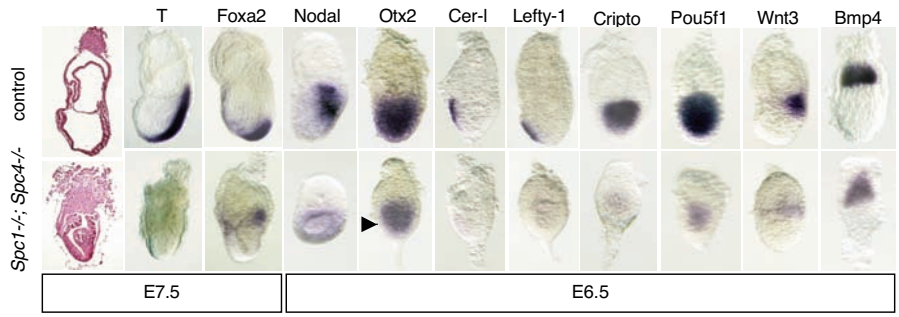

Fig. 3. *Similar to Nodal-deficient embryos, compound mutants lacking Spc1 and -4 (bottom) fail to form mesoderm and endoderm, which are marked in control litter mates (top) by the expression of Brachyury (T) and Foxa2. Nodal eventually is still expressed, but it fails to become repressed on the prospective anterior pole (left). This is due to the lack of a functional AVE (arrowhead) marked by the expression of Otx2, Cer-l and Lefty-1. Also in the epiblast and extraembryonic ectoderm, Nodal fails to maintain expression of known downstream effector genes.*

Processed Nodal can substitute for the lack of Spc1/4 activities

Based on the above experiments, it remained unclear whether Spc1/4 activities directly stimulate Nodal precursor cleavage, or whether they activate the Nodal pathway indirectly, e.g. via an unknown substrate in the ExE that might be required to induce Cripto. To distinguish between these possibilities, embryos were collected on day E5.5 and cultured for 24 hr with or without the ExE. As shown in **Figure 4**, expression of a Nodal.lacZ reporter allele is maintained both in whole embryos and in isolated epiblast explants, whereas induction of Nodal target genes such as Cripto is abolished upon removal of the ExE. More importantly, processed recombinant Nodal produced in stably transfected HEK293T cells is sufficient to restore Cripto expression in such explants, whereas an SPC-resistant mutant Nodal precursor is not. These results are consistent with a model in which the extraembryonic source of convertase activities is required to cleave Nodal in adjacent embryonic tissues (**Fig. 4**). This model places Spc1 and –4 at the top of a complex signaling network, which orchestrates germlayer formation and gastrulation movements using negative and positive feedback regulators such as Lefty and Cer-l, or Cripto, respectively.

Conclusion

Spatial compartmentalization of the expression domains of Nodal and its convertases may result in localized proteolytic cleavage and hence the establishment of a gradient of Nodal activity that is instrumental to pattern the entire conceptus already before gastrulation. In addition, it might serve as a

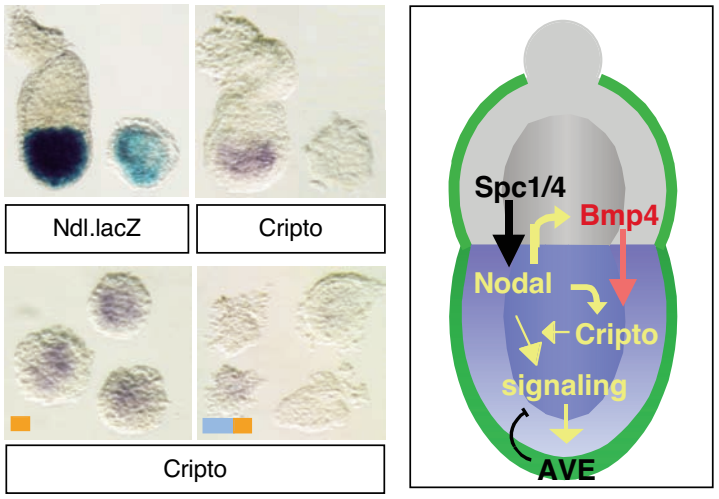

Fig. 4. *In cultured embryo explants, a Nodal^{lacZ} reporter allele is expressed independantly of the extraembryonic ectoderm. However, Nodal can induce its own co-receptor Cripto only if the ExE is present (top). The effect of ExE on Cripto can be mimicked by incubating isolated epiblast explants with processed recombinant Nodal (orange box), but not with uncleaved precursor (bottom), confirming our prediction that endogenous Nodal is inhibited at the level of proteolytic maturation. This suggests that the extraembryonic source of Spc1/4 patterns the embryo through its non-cell autonomous effect on the Nodal precursor (right).*

safety measure to ensure that neither the epiblast nor the ExE will be exposed to Nodal signals unless both tissues develop in a coordinate fashion. Indeed, it seems likely that the activation of Nodal must be tightly limited both temporally and spatially to prevent ectopic induction of bona-fide oncogenic factors such as Cripto, Wnts and Fgfs. To be sure, we only begin to appreciate that the concerted action of these signaling pathways is critical for normal tissue homeostasis. It seems safe to predict, therefore, that many interesting surprises still await us concerning their mechanism of action in normal cells, and under what circumstances tumor cells might hijack this pathway to their own advantage.

Acknowlegdement

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References

Beck, S., Le Good, J. A., Guzman, M., Haim, N. B., Roy, K., Beermann, F., and Constam, D. B. (2002). Extraembryonic proteases regulate Nodal signalling during gastrulation. Nat Cell Biol 25, 25.

Brennan, J., Lu, C. C., Norris, D. P., Rodriguez, T. A., Beddington, R. S., and Robertson, E. J. (2001). Nodal signalling in the epiblast patterns the early mouse embryo. Nature 411, 965-969.

Constam, D. B., Calfon, M., and Robertson, E. J. (1996). SPC4, SPC6, and the novel protease SPC7 are coexpressed with bone morphogenetic proteins at distinct sites during embryogenesis. J Cell Biol 134, 181-191.

Constam, D. B., and Robertson, E. J. (1999). Regulation of bone morphogenetic protein activities by pro domains and proprotein convertases. J Cell Biol 144, 139-149.

Constam, D. B., and Robertson, E. J. (2000). Tissue-specific requirements for the proprotein convertase Furin/SPC1 during embryonic turning and heart looping. Development 127, 245-254.

Dubois, C. M., Laprise, M. H., Blanchette, F., Gentry, L. E., and Leduc, R. (1995). Processing of transforming growth factor beta 1 precursor by human furin convertase. J Biol Chem 270, 10618-10624.

Lu, C. C., Brennan, J., and Robertson, E. J. (2001). From fertilization to gastrulation: axis formation in the mouse embryo. Curr Opin Genet Dev 11, 384-392.

Norris, D. P., Brennan, J., Bikoff, E. K., and Robertson, E. J. (2002). The Foxh1-dependent autoregulatory enhancer controls the level of Nodal signals in the mouse embryo. Development 129, 3455-3468.

Perea-Gomez, A., Vella, F. D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H., Behringer, R. R., and Ang, S. L. (2002). Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. Dev Cell 3, 745-756.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature 397, 707-710.

Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., Le Meur, M., and Ang, S. L. (1998). Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. Development 125, 845-856.

Roebroek, A. J. M., Umans, L., Pauli, I. G. L., Robertson, E. J., van Leuven, F., Van de Ven, W. J. M., and Constam, D. B. (1998). Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. Development 125, 4863-4876.

Shawlot, W., and Behringer, R. R. (1995). Requirement for Lim1 in head-organizer function. Nature 374, 425-430.

Thomas, P., and Beddington, R. (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. Curr Biol 6, 1487-1496.

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