Miniproposal

A Conserved Role of Cas-Spg System in Endoderm Specification during Early Vertebrate Development

Background: How multipotent early embryonic cells differentiate into endodermal lineage remains elusive. Although a group of transcription factors are implicated in endoderm development, how they work together to induce endoderm differentiation is largely unknown. In zebrafish, Spg, a POU domain transcription factor, acts with Cas, a prospective endodermal HMG domain transcription factor induced by Nodal (TGF-beta superfamily) signaling, to activate endoderm development through activation of endoderm specific genes such as sox17(Reim et al., 2004). In mice, chimeric study showed that Smad2-mediated TGF-beta signaling(Tremblay et al., 2000) and Sox17(Kanai-Azuma et al., 2002) are also required for the initial specification of the endoderm. Oct4, mice orthology of zebrafish spg, controls lineage commitment in a dose-dependent manner: an increase in expression causes differentiation into primitive endoderm and mesoderm, while downregulation induces dedifferentiation to trophectoderm(Niwa et al., 2000). POU and SOX(SRY-related HMG) transcription factors have been suggested to exemplify their functional partnership through combinational binding onto Sox-Oct enhancer element(Remenyi et al., 2003). Whether there exits a functional cas homolog in mice that acts with Oct4 to induce endoderm differentiation awaits investigation.

Preliminary Studies: Research in our lab using ES cell line stably transfected with nkx2.5(cardiac-specific transcription factor) promoter:GFP reporter construct and sox17(endoderm specific transcription factor) promoter:CFP reporter construct showed that upon Nodal treatment, Oct4 mRNA and protein expression levels are transiently upregulated(24hr and 48 hr but not after); Sox7 mRNA level can hardly be detected before Nodal induction, but started to express at 24hr till 96hr post Nodal treatment; Expression of both reporter genes can be observed in a portion(>20% each) of the differentiated ES cells(96hr). In an attempt to identify transcription factors that play essential role in endoderm formation, we initiated an siRNA screening using a series of short interfering RNA expression vectors targeted to various transcription factors and transfected them into the ES cell line. Among the transcription factors tested, Sox7 siRNA suppressed the sox17:CFP expression without significantly affecting nkx2.5:GFP expression. Immunostaining of the ES cells upon Nodal treatment(24 and 48hr) showed that Sox7 colocalize in the nuclei of a portion of the ES cells with Oct4 staining. Immunostaining of the mice blastocyst section further confimed that Sox7 colocalizes in the nuclei of a portion of the cells expressing Oct4 in the ICM. Coimmunoprecipitation experiments performed in Nodal induced ES cells(48hr) suggests that Oct4 and Sox7 associate in the same complex.

Based on the clue from the zebrafish cas-spg system and our preliminary data in mice, we hypothesize that ***Sox7 acts with Oct4 to induce endoderm differentiation in mice early embryonic development.*** To test the hypothesis, we propose the following specific aims:

Aim 1: ***To test the role of Oct4 and Sox7 together for mice endoderm specification.***
Aim 1a: *Examine if Sox7 and Oct4 are both required for endoderm specification*

Rational: Oct4 protein is abundant in the ICM of early blastocysts and downregulated in trophectoderm, while even more highly expressed in nascent primitive endoderm(Palmieri et al., 1994). Our preliminary data suggested that Nodal induced mesoendoderm differentiation

correlated with Oct4 upregulation. And siRNA knowdown of sox7 repressed the endodermal fate, suggesting that Sox7 is necessary in this lineage specification. However, it is not clear whether Oct4 upregulation is required for endoderm differentiation or merely for mesoderm differentiation. Also, it is not clear whether loss of Sox7 affects endodermal specification in vivo.

Approach: To test if transient Oct4 upregulation is required for endoderm differentiation, siRNA or antisense cDNA mediated Oct4 knockdown will be performed in Nodal induced ES cells expressing nkx2.5 promoter:GFP and sox17 promoter:CFP. Knockdown efficiency will be verified by qPCR and Western blotting. Empty vector transfected cells will serve as control. GFP and CFP signals will be monitored by fluorescent microscopy and quantified by flow cytometry. Other markers for endoderm and mesoderm specific genes will also be tested by qPCR.

To examine if loss of Sox7 affects endodermal specification in vivo, we will generate $sox7^{-/-}$ ES cells carrying reporter transgene sox17 promoter:CFP and inject it into blastocyst to create chimera mice. Wildtype ES cells with the same reporter gene will serve as control.

Anticipated results and potential problems: Oct4 knockdown level should be adjusted so that there will be similar level of Oct4 expression as is before Nodal induction. Loss of nkx2.5:GFP expression will also serve as control, since transcient Oct4 upregulation has been suggested to be required for cardiac gene expression. If sox17:CFP expression is also repressed, it suggested that transcient Oct4 upregulation is required for endoderm specific gene expression. However, if the expression of reporter gene is not downregulated, it suggests that sox17 is regulated through another pathway independent of Oct4. We will further look at expression of other endoderm markers to see if they are related to transcient Oct4 upregulation. If none of them are affected, it suggests that that transcient Oct4 upregulation is not required for endoderm specification. If some of the markers are even upregulated, it is also interesting, suggesting that Oct4 plays a role in differentiating mesoderm and endoderm.

For the $sox7^{-/-}$/sox17:CFP donor and $sox7^{+/+}$ recipient chimera mice, we expect to see the loss of sox17:CFP reporter expression and loss of endoderm germ layer compared with the control, suggesting Sox7 is required for endoderm specific gene expression and endoderm formation. If the sox17:CFP reporter expression is too low in the wildtype control, we will alternatively use siRNA of sox7 to inject into blastocysts and reimplant into pseudopregnant mice to see if endoderm development will be affected. If we can also observe GFP expressing in the $sox7^{-/-}$ doner chimera mice, it suggests that sox17 can be activated through other pathways in vivo, probably independent of Nodal signaling. We may further test this possibility by using Nodal antagonists.

Aim 1b: *Examine if Sox7 and Oct4 are sufficient for endoderm specification*

Rational: It has been suggested that misexpressiong of cas can ectopically elicit endoderm differentiation in an spg background but cannot do so without spg. It would be interesting to test whether ectopic expression or forced overexpression of Sox7 will induce endoderm specification and whether that is Oct4 dependent.

Approach: To test if upregulation of Sox7 with Oct4 can induce endoderm specific gene expression, we will use Tetracyline(Tc) inducible transgene of Sox7. We will withdraw Tc upon Nodal induction to co-activate Sox7 expression. Non Nodal treated cells and non Tc withdrawn cells will serve as control.

Anticipated results and potential problems: Since the function of a given transcription factor might be dose-dependent, the Sox7 transgene expressing level in non Nodal induced Tc withdrawn cells be adjusted comparable to the Sox7 expression level in Nodal induced non Tc withdrawn cells. Then the same condition of Tc withdrawn will be applied to Nodal induced cells and see if Sox7 transgenic cells show higher level of sox17:GFP reporter gene expression compared with the non Tc withdrawn cells. If sox7 transgene upregulation and Nodal induction promotes endoderm specific gene expression compared with Nodal induction alone, it suggests that Sox7 and Oct4 are sufficient for endoderm specification. If sox7 transgene upregulation alone, without Nodal induction can drive endoderm specific gene expression, it suggested that sox7 acts downstream or independent of Oct4 for endoderm specification. To further test if Sox7 can induce endoderm formation in vivo, ES cells overexpressing Sox7 will be transplanted into mice ICM or trophectoderm and do lineage tracing of the transplanted cells to see if they can ectopically express endoderm marker.

Aim 2: ***To test the regulatory mechanism of Oct4 and Sox7 in endoderm differentiation.***
Aim 2a: *Examine whether Oct4 and Sox7 interacts in a complex on the enhancer of endoderm specific genes*

Rational: In zebrafish, Spg and Cas has been shown to be able to synergistically activate sox17 promoter. Immunostaining of the mice ES cells and blastocyst suggested colocalization of Oct4 and Sox7. Coimmunoprecipitation experiments suggests that Oct4 and Sox7 associate in the same complex in vivo. However, it is not clear whether these two proteins interact through direct binding, if they form a complex that binds DNA and whether sox17 promoter is one of their shared target.

Approach: GST-pull down will be performed using purified GST-Oct4 and HA-Sox7 to test they can interact directly in vitro. EMSA assay using Myc-Oct4 and HA-Sox7 affinity purified from transfected cell culture will be performed to test if they can cause the mobility shift of the DNA containing Octamer and Sox elements. The specificity of the binding will be confirmed by supershift analyses with anti-Myc and anti-HA antibodies. Chromatin immunoprecipitation assay using Oct4 and Sox7 antibody in ES cells 48hr after Nodal treatment will be performed to see if they co-occupy the endoderm specific gene promoter such as sox17.

Anticipated results and potential problems: If GST-Oct4 can pull down HA-Sox7, it suggested direct interaction between the two proteins. Further mutation of the POU and HMG domain will test whether the interaction is via these domains. If we observe mobility shift in Myc-Oct4 and HA-Sox7, it suggests the binding of the two factors to DNA. We can further mutate the consensus Oct or Sox binding site to see if it will restore the mobility shift. If the purified protein cannot cause the mobility shift, it is possible that the binding requires other partners. To test this we will add ES cell lysates and see if it helps in the mobility shift. To better test the binding of the two factors to DNA in vivo, ChIP assay will be performed to test if they co-occupy the

endogenous gene promoter. Sox17 promoter and other known endoderm specific gene promoters will be candidate target DNA. Furthermore, ChIP on Chip analysis will be performed in Nodal induced ES cells and see how the two transcription factors localize to the genomic region.

Aim 2b: *Examine whether Oct4 and Sox7 activate or repress each other*

Rational: Our preliminary data suggests that Oct4 is transiently upregulated and then turned down during mesoendoderm differentiation, while Sox7 is turned on. Although in zebrafish, Spg and Cas seem to cooperate in activation of sox17 expression, it is unclear if in mice early embryonic development, these two factors cooperate with each other in transcriptional activation of endoderm specific gene expression, or one may repress the activity or expression of another.

Approach: Co-transfect oct4 and/or sox7 with sox17 promoter driven luciferase reporter DNA into mice ES cells to see if they can synergistically activate the endoderm specific gene promoter. Furthermore, test the luciferase reporter activitity using Oct4 and Sox7 promoter.

Anticipated results and potential problems: If both Sox7 and Oct4 are required to activate sox17:GFP expression, it suggests that these two factors act synergistically. If Sox7 alone activates sox17:GFP expression, which will be repressed with Oct4 co-expressed, it suggests that Oct4 acts as an co-repressor for the endoderm specific gene expression. Further experiment using ChIP assay to test Sox7 or Oct4 localization on the endogenous sox17 promoter may be performed at different time points to test if Oct4 recruits Sox7 to the target promoter but later on gets off the promoter, which allows target gene activation by Sox7. Also, testing the Sox7 and Oct4 transcriptional activity on Oct4 and Sox7 promoter will allow us to investigate the possible feedback loop between the two factors.

Significance: It is our belief that investigating the conserved role of zebrafish cas-spg system in mice will not only give insight into the evolution of early embryonic development but also have therapeutic implication as to coax stem cells to form endodermal tissue, a likely prerequisite to forming endodermal organs de novo.

References:

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