Published in final edited form as:

Genesis. 2012 October ; 50(10): . doi:10.1002/dvg.22038.

Defining progressive stages in the commitment process leading to embryonic lens formation

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Summary

The commitment of regions of the embryo to form particular tissues or organs is a central concept in development, but the mechanisms controlling this process remain elusive. The well-studied model of lens induction is ideal for dissecting key phases of the commitment process. We find in *Xenopus tropicalis*, at the time of specification of the lens, i.e. when presumptive lens ectoderm (PLE) can be isolated, cultured and will differentiate into a lens, that the PLE is not yet irreversibly committed, or determined, to form a lens. When transplanted into the posterior of a host embryo lens development is prevented at this stage, while approximately 3 hr later, using the same assay, determination is complete. Interestingly, we find that specified lens ectoderm, when cultured, acquires the ability to become determined without further tissue interactions. Further, we show that specified PLE has a different gene expression pattern than determined PLE, and that determined PLE can maintain expression of essential regulatory genes (e.g. *foxe3*, *mafB*) in an ectopic environment while specified PLE cannot. These observations set the stage for a detailed mechanistic study of the genes and signals controlling tissue commitment.

Keywords

specification; determination; eye; *Xenopus tropicalis*

Introduction

During development, the commitment to make a particular tissue or organ is typically made long before overt differentiation. Embryonic commitment is presented as a central concept in developmental biology (Gilbert, 2010; Wolpert and Tickle, 2011) and yet its mechanism remains largely an enigma. It has been argued that specification is an early phase of this commitment process, i.e. when specified tissue is placed in culture, it can go on to differentiate without further tissue interactions. However, cell fates within the tissue at this phase may still be altered when the tissue is placed in an environment providing strong counter-inducing signals e.g. in ectopic locations in the embryos. In contrast, during the later phase of commitment, determination, cell fates are irreversible wherever the tissue is placed.

Although the distinction between specification and determination has long been proposed (Slack, 2006), there is relatively little experimental data substantiating this conceptual framework. Studies on neural determination indicate that the posterior neuroectoderm is transiently specified to form anterior neural tissue (Eyal-Giladi, 1954; Saha and Grainger, 1992; Sive *et al.*, 1989). More recently, there have been a few studies on tissue or organ formation that support the concept of these two different phases of commitment (Auda-Boucher *et al.*, 2000; Baker *et al.*, 1999; Groves and Bronner-Fraser, 2000; Saito *et al.*,

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2002; Wilson *et al.*, 2000). In these studies there are, however, often limitations in the manner in which the stages in the commitment process can be examined because either one cannot follow all stages of the commitment process in a given system, or there are challenges in culturing tissues that interfere with assaying developmental phenomena.

We have been examining these issues during the development of the amphibian embryonic lens where, in contrast to many other model organisms, it is feasible to examine all stages of development, and to readily study developmental commitment in cultured tissues or in transplants (Grainger, 1992; Grainger *et al.*, 1997). We have described a sequence of events in lens formation that serves as a framework for the studies presented here. The lens commitment process begins with a short period during gastrulation, between *Xenopus* stages 10 and 11 (Nieuwkoop and Faber, 1994) when animal cap ectoderm is competent to form lens, followed by a “bias” stage for lens formation in the presumptive lens ectoderm (PLE) at the neural plate stage (stage 14), and then specification of the PLE at the neural tube stage, *Xenopus* stage 18. The term bias refers to the property of the PLE at the neural plate stage whereby it appears to have received an initial inductive signal allowing it to be very easily responsive to later inductive signals, but it is not yet specified. Differentiation of the lens begins about ten hours later at the tailbud stage (stage 28) when lens-specific crystallin proteins are first synthesized. However, we had never tested whether specified ectoderm is determined, or whether this occurs after stage 18, framing the issue we address in this paper.

In parallel to the commitment question, as assessed by biological parameters, is the issue of the genetic basis for commitment. A substantial number of transcriptional regulators is activated either in the PLE or throughout the placodal tissues (e.g. ear, lens and olfactory regions) during the stages described above (Ogino *et al.*, 2012). A few key transcription factors are introduced here that were monitored in the experiments in this study since they are likely to play particularly key roles in the commitment process, though the connection between a gene set and the commitment process has yet to be established.

Among the earliest of the transcriptional regulators activated in the PLE is the central eye regulator, the homeodomain-containing transcription factor *pax6*, already active at the neural plate (bias) stage (Zygar *et al.*, 1998). A few hours later, at the time of specification, one of the few genes identified as newly transcribed in the PLE at this stage is the winged helix transcription factor *foxe3* (Kenyon *et al.*, 1999). This gene is transcribed as a downstream response to a Notch-mediated lens-inducing signal originating from the neural plate or optic vesicle (Ogino *et al.*, 2008), and thus is a gene which may play a critical role in lens commitment. Just after PLE specification, the HMG-box transcriptional regulators *sox3* (Zygar *et al.*, 1998) and slightly later, *sox2*, are activated in the PLE in *Xenopus* after neural tube closure but before lens differentiation, and are known to play a key role in crystallin gene activation in concert with *pax6* (Ogino *et al.*, 2012). Maf family transcriptional regulators are also activated just after neural tube closure in *Xenopus*, first *mafb* and then *l-maf*, and both are known to play key roles in controlling lens differentiation in *Xenopus* (Ishibashi and Yasuda, 2001). Lens differentiation begins with the activation of γ -crystallin at stage 28 and is assayable in a transgenic reporter line in *Xenopus tropicalis* in which the promoter from this gene is linked to GFP, providing a very rapid assay for lens differentiation in our studies here (Offield *et al.*, 2000). *X. tropicalis*, because of its more rapid life cycle, is readily suited to making such transgenic lines, and while embryos are somewhat smaller than the widely used *Xenopus laevis* embryos, are nonetheless highly suitable for embryological manipulations (Harland and Grainger, 2011; Hirsch *et al.*, 2002).

In the experiments presented here we have found that specified PLE (stage 18) is not yet determined, but only a few hours later (stage 21) it becomes determined as monitored by transplantation assays. We also show that specified PLE behaves differently from

determined PLE in having a different expression pattern of key lens genes when cultured, and in turning off several key lens genes in transplants that are maintained in determined ectoderm. Further, we show that the transition from lens specification to lens determination is a tissue autonomous process.

Results

Stage 18 PLE is specified but not determined

Since specification may be an earlier phase of commitment than determination, we wanted to examine whether specified PLEs are or are not determined. This was tested by examining whether lens differentiation occurs in specified PLE when placed in different environments within a host embryo.

Before undertaking this study, we first verified the time of specification of the PLE. Previous studies in *Xenopus laevis* showed that the PLE is specified at stage 19, the last stage before completion of neural tube closure (Henry and Grainger, 1990). Here, using an established transgenic *Xenopus tropicalis* reporter line with a 2.2kb γ 1-crystallin promoter driving GFP3 expression, greatly facilitated the scoring of lens differentiation by observing the appearance of GFP (Offield *et al.*, 2000). PLEs on the right side of embryos from transgenic frogs were explanted. PLEs on the left side of the donor were left intact as a control. Both the explant and the donor were cultured in parallel for about 48 hours (until donors reached stage 41) and then the GFP fluorescence from the crystallin transgene was assayed in the explants as an indication of lens differentiation (Figure 1). The results showed that the PLEs from embryos stage 18 and older could differentiate when isolated and cultured in a high percentage of cases (typically greater than 70% in many separate experiments).

A transplantation assay was used next to examine whether newly specified PLE is also determined. PLEs on the right side of stage 18 donor embryos from transgenic frogs were transplanted into different locations of stage 18 host embryos. PLEs were transplanted into one of 12 regions in a stage 18 embryo host (illustrated in Figure 2a). After culture to stage 41, GFP fluorescence from the crystallin transgene was scored in the PLE transplant. Host embryos were also transgenic, serving as a control for the onset of transgene expression (denoted with white arrowheads in Figure 2; expression in the hindbrain, typical of γ -crystallin expression in *Xenopus* (Smolich *et al.*, 1994) is also noted with an arrowhead). Lens differentiation was recorded in each of these 12 different regions, three of which are illustrated in Figure 2. When transplanted into anterior or dorsal regions such as A1, B1, C1 and A2, lens differentiation in the transplanted PLE occurred in a very high percentage (>90%, Figure 2b and 2b') and with very strong fluorescence (+++). In contrast, when transplanted into the posterior or ventral regions of stage 18 embryos such as C2, A3, B3, C3, A4, B4 and C4, positive GFP expression occurred in a very low percentage (<30%) of cases. Even when the PLE could differentiate in these posterior or ventral regions, the GFP fluorescence of γ -crystallin gene was very weak as shown in Figure 2d'. The percentage of positive cases of lens differentiation in the PLE when transplanted into the central region of an embryo was intermediate, and the strength of the GFP in this case was intermediate as well. When compared to the explant results (69% positive, Figure 1), the transplant results indicate that the posterior and ventral embryonic environment of stage 18 embryos are inhibitory environments for lens differentiation in newly specified PLE (summarized in Figure 2e), and thus, that the PLE is not determined at this stage. However, the possibility that there are inhibitors of lens differentiation in the posterior and ventral regions of the embryos which lead to the absence of lens formation in the corresponding regions *in situ* cannot be completely ruled out from these experiments. Results from the next section, however, indicate that negative responses in posterior and ventral regions are due to the

inability of the transplants to undergo transition from specification to determination rather than the inhibition of lens differentiation.

The PLE of *Xenopus tropicalis* is determined at Stage 21

Since the PLE at the stage when it is first specified does not appear to be determined, this led us to identify a stage when the PLE attained this more definitive stage in the commitment process. Taking the posterior region B4 of stage 18 embryos as a test site, PLEs on the right side of embryos ranging from stage 18 to stage 22 were transplanted into this region. PLEs on the left side of donors were left intact as controls. The GFP fluorescence from the crystallin transgene in the transplanted PLE was recorded 36 hours later when hosts had reached stage 36 (Figure 3). The results showed that from stage 21 onward, the PLE would inevitably differentiate in the B4 region. To confirm that stage 21 PLE is determined throughout the embryo, heterotopic transplants were performed again into the 12 previously described regions in the stage 18 embryo (Figure 4). The results showed that the stage 21 PLE could differentiate into lens no matter where it was transplanted (100% in every region, Figure 4a, b, and c). These data support the conclusion that stage 21 PLE is determined and also imply that negative results seen in transplants of stage 18 PLE to the posterior were not due to an inhibitor of lens differentiation, but because the stage 18 PLE must be still labile with regard to its determination status.

The transition from lens specification to lens determination is a tissue autonomous process

Previous work has clearly demonstrated the requirement for inductive interactions in eliciting specification in the PLE. The identification of a slightly later stage when determination of the PLE occurs led us to ask whether the transition from the lens specification status to the lens determination status also requires induction, or whether it is a tissue autonomous process. To test these alternatives, the heterotopic transplant test was performed using specified PLEs that were aged *in vitro* to the stage when the PLE would be expected to be determined (Figure 5). Thus, PLEs on the right side of embryos at the lens specification stage (stage 18) were explanted and cultured *in vitro* until the donor embryo reached the determined stage (stage 21, Figure 5a). The cultured PLEs were then transplanted into region B4 (Figure 5b) or region C3 (Figure 5c) of stage 18 hosts to test whether lens differentiation of cultured PLEs could occur. The results of these transplants showed that the short period of culture is sufficient to turn the specified PLE into determined PLE, since transplanted PLE differentiated into lenses either in the posterior region (100%, N=8) or the ventral region (100%, N=6) both regions which strongly inhibit lens formation in specified ectoderm. This implies that the transition from specified PLE to determined PLE requires no further extrinsic signals, that the transition from lens specification to lens determination is a tissue autonomous event.

Comparison of gene expression profiles in cultured PLE isolated at different stages during the commitment process

Since stage 18 (specified) PLE shows a different responsiveness than stage 21 (determined) PLE in some embryonic environments, we investigated whether these two tissues would show differences in terms of expression of key early lens genes when cultured to a stage when lens differentiation would occur in order to begin to assess molecular correlates to the biological transition to determination. In addition to comparing expression data from cultured stage 18 and 21 PLE, to add another assay point for examining molecular correlates of commitment, PLEs from stage 14 embryos were also explanted and cultured to investigate what genes are activated or remain activated in PLEs that are “biased” toward lens formation but cannot form a lens when cultured. To address these questions, we used a

real-time PCR assay to quantitatively examine the expression levels of six early lens genes, as well as *γ-crystallin*, in control tissues (isolated directly from the embryos) as well as cultured PLE removed from the embryo at stages 14, 18 and 21 and cultured to stage 36. The results are shown in Figure 6.

Beginning with the endpoint assay, *γ-crystallin* activity (Figure 6g), it is clear that both specified and determined PLE express the member of the *γ-crystallin* gene family being assayed here, but clearly the determined tissue has a much higher level of expression (and cultured stage 14 biased PLE does not express detectable levels of this gene, as expected). One might consequently anticipate that either higher levels or a more complete set of lens-activating genes are found in determined PLE. Indeed, for example, *mafb*, known to be important in crystallin gene activation in *Xenopus*, is more strongly expressed in determined PLE (Figure 6c). Interestingly, *l-maf* is not detectably expressed in any of the cultured tissues (Figure 6d) and therefore cannot be essential for crystallin gene expression. Since *l-maf* is required for proper lens fiber cell differentiation (Ishibashi and Yasuda, 2001) this later event in lens differentiation is not likely to be occurring in these explants, consistent with our observations on the morphology of the lentoids and small lenses that form in culture (Henry and Grainger, 1990). The optic vesicle is required for *l-maf* expression in the PLE (Ishibashi and Yasuda, 2001). These results illustrating the failure of *l-maf* to be activated in cultured PLEs, even determined PLEs, also implies that the short period of time, from stage 18 to stage 21, during which time the PLE is first in contact with the underlying optic vesicle, is not sufficient to trigger *l-maf* expression in the lens. Levels of *foxe3* and *pax6* (Figure 6b and 6a, respectively) are not significantly different between specified and determined PLE explants.

Expression levels of *sox3* and *sox2* (Figures 6f and 6e, respectively) were assayed here because of the proposed roles of these genes as co-activators of crystallin genes in conjunction with *pax6*, as discussed earlier. The expression patterns are somewhat complicated by the fact that these two genes, while not expressed in the PLE before stage 21, are expressed in nearby dorsal lateral placode (Zygar *et al.*, 1998) and were likely detected in our assays of PLEs directly removed from the embryo at stages 14 and 18 because parts of the dorsal lateral placode tissue were included in dissected PLE regions. As might be expected, *sox3* is seen in stage 21 PLE since it is known to be expressed in this tissue in vivo. However in cultured ectoderm no expression of these two genes is discerned at any stage, suggesting that *sox2* and *sox3* expression is not obligatory for activation of crystallin genes and likely requires further tissue interactions between the PLE and optic vesicle.

The gene activity in cultured, biased PLEs reveals that some early lens genes are active in this tissue, but because it is not yet specified, they cannot be sufficient to activate the lens program. While crystallin is not synthesized, since the tissue is not specified at this stage, *pax6* is maintained in cultured stage 14 PLE and *mafb* is activated almost to the same level as seen in specified PLE explants. This suggests that *mafb*, while known to be an activator of crystallin genes, cannot be sufficient to elicit a lens-forming response. Interestingly, *foxe3* is not activated at detectable levels in these assays and may then be an essential factor in specification and determination.

In summary, biased PLEs express some key early gene regulators when cultured to lens differentiation stages (e.g. *pax6* and *mafb*), but results show that other genes are not activated (e.g. *foxe3*) and thus may be required for the transition to specification. Both specified and determined PLEs do not activate several genes known to play a role in lens differentiation (*sox2*, *sox3* and *l-maf*) but which cannot be required for the initial stages of lens differentiation assayed in our experiments.

Gene expression analysis in specified and determined PLE transplanted to the posterior of a host embryo

Although the ability of newly specified PLE and determined PLE to differentiate in culture is similar, they clearly respond very differently when they are transplanted into an unfavorable embryonic (posterior or ventral) environment. Therefore, the next question we investigated was whether the PLEs transplanted at these two different stages would show different spectra of gene expression, besides crystallin, when placed into the “unfavorable” embryonic environment in the posterior of a stage 18 embryo that prevents lens formation in stage 18 PLE. Specified stage 18 PLEs and determined stage 21 PLEs from donor embryos injected with fluoro-ruby as a lineage tracer were transplanted into the B4 region (Figure 7a and 7b). Whole mount *in situ* hybridizations with probes for *pax6*, *foxe3*, *mafb*, *sox3*, *sox2* and *l-maf* were conducted on host embryos at stage 36. Pictures of host embryos taken in bright field were superimposed on the pictures of host embryos taken to reveal lineage label fluorescence (Figure 7c) to be able to superimpose the site of implanted tissue and target gene expression.

Gene expression in transplants of stage 18 PLE and stage 21 PLE is illustrated in Figure 7 where one can see striking differences between the two kinds of transplants. Transplants of stage 18 PLE assayed when the host has reached stage 36 show loss of initial expression of *pax6* and *foxe3* (d and e in Figure 7), two early lens marker genes. Only 23% of the transplants examined showed the staining of *pax6* (Table 1). This is consistent with the previously described phenomenon that a very small fraction of the transplants showed lens differentiation in the posterior region of stage 18 embryos (Figure 2). No *mafb* could be detected in stage 18 PLE transplants (f in Figure 7, and Table 1) though previously we showed that when cultured alone, *mafb* is clearly strongly increased in stage 18 PLE explants, and even in stage 14 unspecified PLE explants (Figure 6c). To further examine the intermediate temporal changes in gene expression in transplanted tissues, the hosts of heterotopic transplants of stage 18 PLE were fixed at stage 21 and whole mount *in situ* hybridization showed that *mafb* transcript can be transiently detected at this intermediate stage (r in Figure 7 and Table 1). This suggests that the inhibitory embryonic environment leads to repression of at least some genes initially activated in the PLE. Moreover, *l-maf* expression is not generally activated in transplanted stage 18 PLE (i in Figure 7, and Table 1). Similar to *pax6*, a small fraction (17%) of transplants show weakly positive staining for *l-maf* (Table 1). For *sox2* and *sox3* expression in stage 18 PLE transplants, although there was some staining of *sox2* and *sox3* around the transplanted area, this staining was irrelevant to this study since it was not in the labeled transplanted PLE (g, h, s and t in Figure 7). In short, all the genes examined were either not expressed or were completely or largely extinguished in transplants of specified stage 18 PLE. In contrast, stage 21 PLEs maintained the expression of *pax6*, *foxe3* and, with lesser frequency, *mafb*, in the “inhibitory” embryonic environment (j, k and l in Figure 7 and Table 1) and, further, activated the expression of *sox3*, *sox2*, and *l-maf* (m, n and o in Figure 7, and Table 1). The activation of *l-maf* is particularly interesting since this gene is not activated in specified or determined PLE when cultured in isolation. This suggests that there is a factor even in the posterior of embryos that permits a more normal degree of lens differentiation than is seen in cultured ectoderm. The different responses between newly specified PLE and determined PLE suggest that a genetic program, though not fully defined in our studies here, is present in determined PLE but not in newly specified PLE to enable determined PLE to be unresponsive to the potentially fate-changing embryonic environment.

Discussion

We have demonstrated differences in the biological responses of presumptive lens ectoderm taken from embryos at distinct stages of the commitment process and have begun to correlate behavioral properties with gene expression profiles. The different responses of newly specified PLE and determined PLE when placed into the posterior embryonic environment suggest that there are two key levels controlling determination that are highlighted by our work: (1) the intrinsic activity of the network of transcription factors essential for lens formation; and (2) the activities of the extrinsic signaling systems such as the one that blocks determination of specified ectoderm in the “unfavorable” embryonic environments, or the tissue interactions that are required for the progression of biased PLE to the specified state.

Regarding the first point, we can see from our data on gene expression changes in cultured PLEs and transplanted PLEs that there are significant changes in the expression of transcription factors believed to be part of the regulatory network controlling lens commitment. Enough evidence from many sources has accrued so that one can begin to outline components of such a network, summarized in a recent review (Ogino *et al.*, 2012) highlighting the multitude of interactions and feedback loops that are thought to be associated with activation and stabilization of expression of genes like *pax6*, *sox2*, *sox3*, *foxe3* that may be involved in lens commitment, and others that play key roles in ultimately turning on crystallin genes in the lens. In our study we have only examined a few of the many genes involved in this network to begin to get some information about key factors that might be required for determination (and which are then down-regulated in transplants of specified PLEs), or, for example, active, activated, or missing when biased PLE is cultured (and does not form a lens). From this study, it is dramatically clear that when the original program of lens formation in the heterotopic transplants of newly specified PLE is blocked by an “unfavorable” embryonic environment a potent inhibition of already transcribed early lens genes is triggered (most strikingly *foxe3* and *mafb*, but also *pax6*), clearly interrupting the normal gene circuitry which remains largely intact when specified PLE is explanted and allowed to differentiate into at least rudimentary lenses. Even in cultured specified PLEs, the reduction in the levels of some genes (e.g. *mafb*) and failure to activate others (*l-maf*) highlights the need for further tissue interactions with the optic vesicle to stimulate full lens differentiation beyond the “core” responses occurring during specification. Surprisingly, when biased ectoderm is cultured, *mafb* is significantly upregulated, but clearly this is not sufficient to activate the lens-forming program, perhaps because *foxe3* is not concomitantly activated or because *mafb* is not essential for lens formation. In mouse, not all *Maf* family genes are essential for lens differentiation (Takeuchi *et al.*, 2009). But with this initial information one can at least envision that such core elements can be defined by further study of the full complement of genes, e.g. by RNA-Seq, activated during specification or determination stages, or prevented from activation in cultured biased ectoderm. The significance of gene expression changes can be assayed in transplants of specified PLEs, and by adding or inhibiting gene activities to tissues at different stages in the commitment process to manipulate (and thus define) the biological status of biased, specified or determined PLEs with the ultimate goal of defining the genetic machinery that controls how specified lens ectoderm is transformed in a tissue-autonomous manner into a stably determined lens.

The second aspect of the commitment process that will be vital for understanding the determination mechanism will be to clarify which signaling factors in the posterior embryonic environment impinge on the lens determination process. Studies probing the molecular mechanisms of the posterior environment suggest several possibilities. Retinoic acid, TGF- β superfamily members (e.g. BMPs), FGFs and Wnts have all been shown to exist

in high concentrations in the posterior and ventral regions of stage 18 embryos compared to dorsal and anterior regions (Domingos *et al.*, 2001; Kiecker and Niehrs, 2001; Kraft *et al.*, 1994). In addition, there is some evidence showing the suppression of lens formation in the presence of some of these signaling pathways. For instance, the presence of FGF in chick embryos promotes an olfactory fate at the expense of lens fate in the head ectoderm, though by “default”, the whole head ectoderm adopts the lens fate (Bailey *et al.*, 2006). Recent findings concerning the Wnt pathway in the head region indicate that Wnt signaling has an inhibitory role on lens formation (Grocott *et al.*, 2011; Kreslova *et al.*, 2007). As we try to understand the mechanisms of cell determination, the finding that the transition from specification to determination does not require inductive signals also provides the valuable clue that the final step of the commitment process must occur within the PLE itself though this mechanism must also entail changes in responsiveness to external signals.

The significant biological information revealed in the studies presented here presents a valuable platform for future work in untangling the detailed mechanisms of specification and determination. One can anticipate that future work on determination mechanisms will bring to bear the combined strengths of *X. tropicalis* as a model system, with embryological manipulations complemented by the advantages of *X. tropicalis* for genetic and genomic manipulations (Harland and Grainger, 2011).

Methods

Embryonic procedures

Transgenic embryos used were either from an outcross of an F1 generation of a previously established 2.2kb γ -crystallin promoter-GFP3 transgenic line in *Xenopus tropicalis* (Offield *et al.*, 2000) or from an outcross of an F2 homozygous generation of the same line. Wild type embryos used were from an *X. tropicalis* Nigerian F5C line which was bred in the laboratory. Embryos were cultured in 0.1X Modified Barth's Saline (MBS) + gentamicin (Sive *et al.*, 2000). Jelly coats covering the embryos were removed by treatment in 2% free-base cysteine (Sigma) as described (Sive *et al.*, 2000). The vitelline membranes were removed manually just before operation. Dissections were performed in 1× MBS + gentamicin. Tissues or embryos subsequently were raised in 0.5× MBS + gentamicin. All embryos were staged according to normal tables for *Xenopus laevis* (Nieuwkoop and Faber, 1994).

Two types of embryological procedures were performed in this study: PLE explants and PLE transplants. For explants, transgenic embryos from the above described lines served as donors. Donor embryos were first embedded in clay-lined petri dishes. PLEs on the right side of the donor embryos were then removed using a tungsten needle, insuring that no mesoderm or optic vesicle tissue was adherent to isolated PLEs. Isolated PLEs were transferred to 24 well dishes coated with a thin layer of 1% agarose in 0.1× MBS. Donors were transferred to the same dish in different wells and cultured in parallel with the PLE explants for about two days until they reached stage 41. Both donors and PLEs were scored for the occurrence of lens formation by monitoring GFP fluorescence resulting from the crystallin transgene. It was essential to follow donor embryo fluorescence, because in many experiments the transgenic line used was heterozygous and only a fraction of embryos generated were GFP-positive.

For transplant experiments, donors were transgenic embryos, in some cases injected with dextran tetramethylrhodamine (10,000 MW, lysine fixable, fluoro-ruby, Invitrogen) as a lineage label (Sive *et al.*, 2000). Hosts were wild type embryos or crystallin reporter transgenic embryos. Similar to the explant procedure, hosts were first embedded in a clay-lined petri dish. PLEs or other appropriate ectoderm pieces of hosts described in the Results

were removed or discarded. PLEs from donors, which were also embedded in clay, were removed and transferred to the region in hosts from which corresponding ectoderm had been removed previously. A curved glass fragment was pressed on the transplanted PLE for half an hour until the PLE healed on the host embryo. Then both hosts and donors were transferred to 24-well dishes and cultured until donors reached stage 36. A similar scoring method as used for the explants and their donors was used to score for the occurrence of differentiation in the transplanted PLEs. For the experiment which was used to test the tissue autonomous transition from specification to determination, the excised stage 18 PLE was cultured *in vitro* for a period of time until donor embryos reached stage 21 and then this aged PLE was transplanted to the posterior or ventral regions of stage 18 embryos. During the *in vitro* culture period, the PLE has a tendency to roll up and heal into a sphere. To prevent the healing (rolling up) of the cultured PLE, physical force was applied occasionally (every 20 min) to the excised PLE to keep the PLE flat.

Whole mount *in situ* hybridization

Whole mount *in situ* hybridization was carried out according to standard procedures (Sive *et al.*, 2000). Since all the *in situ* hybridizations were carried out on embryos of *Xenopus tropicalis* instead of *Xenopus laevis*, some changes were made to accommodate these embryos. These changes include shorter treatment (8 min vs 10 min) of proteinase K and elimination of RNase treatment for probes when these were made from *Xenopus laevis* cDNA clones. The source of each clone is listed in Supplementary Table 1. Color reaction products were generated using the substrates NBT (Invitrogen) and BCIP (Invitrogen).

Histology and photography

Following *in situ* hybridization and fixation according to (Sive *et al.*, 2000), embryos were examined as whole mounts. Anti-sense RNA *in situ* hybridization probes used in this study are summarized in Supplementary Table 1. Photos of intact embryos were taken through a Zeiss Stemi SV11 fluorescent dissecting microscope and a Zeiss AxioCam MRC5 camera. Under the guideline of clearly representing the data, images, especially GFP images, were processed in Photoshop 7.0 to minimize the difference between the images viewed by camera and those viewed by human eyes. Samples in a given experiment were photographed with the same exposure in order to allow comparisons of relative intensity of GFP fluorescence.

Total RNA extraction and real time PCR

Total RNA from 10 eyes of stage 36 embryos or 10 PLE explants in each experimental group was extracted using Trizol (Invitrogen) following the manufacturer's instruction. For 10 eyes, 500 μ l of Trizol was used. For 10 PLEs, 100 μ l of Trizol was used. cDNA was synthesized from 0.2 μ g of PLE total RNA or 0.5 μ g of stage 36 total RNA in a 20 μ l total volume using the iScript cDNA synthesis Kit (Bio-rad). The cDNA obtained was amplified in the polymerase chain reactions (PCR) in a 25 μ l volume composed of 10 μ l iQ SYBR green supermix, 1 μ l of each primer and 1 μ l DNA template following manufacturer's instruction for the iCycler (Biorad, Cat# 170-8720). Primers used to amplify each gene are shown in Supplementary Table 2.

Real time PCR was monitored by SYBR Green fluorescence. With the additional optical detection system, MyiQ single color real-time PCR detection system (Biorad, Cat#170-9740), the amplified product in each reaction can be monitored in real time and can further be analyzed in MyiQ optical system software version 1.0 (Biorad). All real time PCR reactions ran for 30 cycles of denaturing at 94°C for 30 sec, annealing at the appropriate temperature for 30 sec and extension at 72°C for 30 sec. All primer pairs were designed by using FastPCR software (<http://primerdigital.com/fastpcr.html>). The primer pairs and the

corresponding annealing temperature used for amplifying each gene are summarized in Supplementary Table 2.

When comparing different expression levels of different types of explants, the expression level of stage 36 eye tissue was set as 100%. A serial dilution of cDNA from stage 36 eye tissue was used to make standard curves for each gene. A percentage of expression level was shown after sample tissue was scaled to a standard curve, normalized by expression level of 28S rRNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank lab members Margaret Fish, Takuya Nakayama and Matt Etzell for helpful discussions, and Rollin Reeder for technical assistance. The intellectual contributions provided by former lab members Jonathan Henry, Lyle Zimmerman and Hajime Ogino are also gratefully acknowledged

Work funded by NIH grants EY019000 and EY017400

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Donor stage	PLE position	Number examined	Positive cases (%)	Representative cases
stage 16		24	0
stage 18		50	34 (68%)
stage 21		11	11 (100%)
stage 23		16	16 (100%)

Figure 1. Lens-forming ability of isolated presumptive lens ectoderm. Explants were taken from staged embryos (Nieuwkoop and Faber, 1994) and scored when donor embryos reached stage 41. Diagrammatic views of the right sides of embryos are shown with dashed boxes indicating the position of the PLE at each stage. Positive cases represent those which showed visible GFP driven by a γ -crystallin promoter. Arrows denote the GFP expression in typical PLE explants shown in the right hand column.

Figure 2.

Assessment of developmental potential of specified PLE in transplant experiments. Panel a shows a diagram of 12 regions in the side of a stage 18 embryo into which transplants were placed. b-d show examples of the different degrees of lens-forming ability in different sites indicated by the level of GFP fluorescence in responding ectoderm. PLEs from stage 18 embryos were transplanted into different locations of embryos at the same stage. Host embryos were scored at stage 41. A strong GFP signal (+++) was similar but still less than the level of endogenous gene expression, and could be detected in the transplanted PLE when placed into region B1 as shown in panel b. A moderate response (++), clearly discernible, but much weaker than those responses in the anterior of the embryo, is illustrated in Figure 1c'. Only a weak GFP signal (+) was seen in the transplanted PLE when placed into region C4 as shown in panel d'. Here the response, though clearly positive, was just discernible. The images shown in this figure, and in other experiments, were photographed with the same exposure, and when transplanted into GFP-labeled hosts, as shown in this figure, more readily compared to host expression to determine relative levels. A summary of responses in all 12 regions is shown in e. In panels b', c' and d', arrows indicate the GFP fluorescence in the transplanted PLE and arrowheads indicate the endogenous GFP in hindbrain and lens of the transgenic embryos.

Donor stage	PLE position	Position in hosts	Number Examined	Positive cases (%)
stage 18			28	6 (21%)
stage 19			21	5 (24%)
stage 20			25	6 (23%)
stage 21			20	20 (100%)
stage 22			12	12 (100%)

Figure 3. Lens-forming ability of PLE from different stage donors placed in the posterior of stage 18 embryos. Transplants were made from donor embryos of the γ -crystallin-GFP transgenic line into wild type hosts. Transplants were scored when hosts reached stage 41. Diagrammatic views of the right sides of donor and host embryos are shown with boxes indicating the transplanted PLEs. The numbers of positive cases represent those which showed visible GFP driven by the γ -crystallin promoter.

Figure 4.

Assessment of the developmental potential of stage 21 PLE in transplant experiments. Stage 21 PLE yielded a positive lens response indicated by fluorescence expression of γ -crystallin-GFP when PLEs were transplanted into any of the 12 regions of stage 18 embryos. The regional designations of the embryo are the same as those shown in Figure 2. Panels a-c show results from PLE transplants into different regions. A strong GFP signal (+++) was detected in the transplanted PLE in all sites. A summary of the data from all cases examined in each of the 12 regions is shown in d. In panels 2a',b' and c', arrows indicate the GFP fluorescence in the transplanted PLE and arrowheads indicate the endogenous GFP in host transgenic embryos.

Figure 5.

The transition from lens specification to lens determination is tissue autonomous. Panel a shows the procedure used in this experiment, i.e. PLEs from stage 18 γ -crystallin- GFP3 embryos were removed and cultured alone until the donor embryos reached stage 21. Then aged PLE was transplanted into region B4 (b), a posterior region of a stage 18 host embryo or into region C3 (c), a ventral region. Host embryos were scored at stage 41. Strong GFP expression could be observed in the transplanted PLE in both regions. In panel b' and c', arrows indicate GFP in the transplanted PLE and arrowheads indicate the endogenous GFP in transgenic host embryos.

Figure 6.

A comparison of expression levels of lens genes in cultured stages 14, 18 and 21 PLE. The fold number of gene expression in each group of explants was obtained by comparison of the gene expression level in explants to that in stage 36 head tissue. All expression levels were normalized to 28S rRNA levels in a given tissue. Expression levels of *pax6* (a), *foxe3* (b), *mafb* (c), *l-maf* (d), *sox2* (e), *sox3* (f), and γ -crystallin (g) in each group of samples is illustrated. The gene expression level of non-cultured stage 14, stage 18 and stage 21 PLE represents the endogenous gene expression in PLE at each corresponding stage. “14 to 36” denotes PLE explants excised at stage 14 and cultured to stage 36. “18 to 36” denotes PLE explants excised at stage 18 and cultured to stage 36. “21 to 36” denotes PLE explants excised at stage 21 and cultured to stage 36. Error bars are standard deviations generated from three independent experiments.

Figure 7.

Gene expression assays in transplanted specified and determined PLE. Panels a and b illustrate how PLEs from embryos injected with fluoro-ruby, a lineage tracer, were transplanted at stage 18 and stage 21 respectively to region B4 of stage 18 embryos. When the host embryos reached stage 36, they were fixed and subjected to whole mount *in situ* hybridization and then photographed. How the pictures were manipulated to merge expression and lineage tracer is shown in c using the embryo shown in panel j. Images of typical cases of gene expression in stage 18 and stage 21 PLEs transplanted to the posterior of embryos are illustrated in panels d-u. Panels d-i show specified PLE (stage 18) transplants raised to stage 36. Panels j-o show results from determined PLE (stage 21) transplants. Panels p-u show specified PLE (stage 18) transplants raised only to stage 21. Determined PLE maintains the expression of *pax6*, *foxe3*, *sox3*, *sox2* and *l-maf*, and to a lesser extent *mafb*, while specified PLE loses expression of all of these genes in most cases. Although positive staining of *sox2* or *sox3* could be observed around the transplanted PLE (g, h, s and t), the exact locations of these staining reveals that these positive tissues are not in the lineage tracer marked transplanted ectoderm. Thus, these positive cases are not relevant to this study. Arrows in j-o and p-r highlight the *in situ* hybridization staining in the transplanted PLE. The dashed circles in d-i and s-u highlight the absence of *in situ* hybridization staining in the transplanted tissue.

Gene expression in stage 18 PLE versus stage 21 PLE transplanted into the posterior region of stage 18 embryos.

Table 1

Transplant cultured to	<i>pax6</i>	<i>foxe3</i>	<i>mafb</i>	<i>sox3</i>	<i>sox2</i>	<i>l-maf</i>
<i>a</i> stage 18 to stage 36	23% (N=13)	0 (N=10)	0 (N=10)	20% (N=10)	17% (N=12)	17% (N=12)
<i>b</i> stage 21 to stage 36	100% (N=10)	100% (N=9)	23% (N=11)	100% (N=10)	100% (N=13)	100% (N=13)
<i>c</i> stage 18 to stage 21	100% (N=10)	89% (N=9)	80% (N=10)	0 (N=8)	0 (N=9)	0 (N=8)

Note: Three types of transplants are shown. % represents percentage of positive-staining cases of the corresponding gene in the transplanted PLE. N represents the total number of cases.

a,"stage 18 to stage 36" denotes that stage 18 PLEs were transplanted into B4 region of stage 18 embryos and the host embryos were then cultured to stage 36 for scoring.

b,"stage 21 to stage 36" denotes that stage 21 PLEs were transplanted into B4 region of stage 18 embryos and the host embryos were then cultured to stage 36 for scoring.

c,"stage 18 to stage 21" denotes that stage 18 PLEs were transplanted into B4 region of stage 18 embryos and the host embryos were then cultured to stage 21 for scoring.