The small leucine-rich repeat secreted protein Asporin induces eyes in Xenopus embryos through the IGF signalling pathway

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ABSTRACT

Small leucine-rich repeat proteoglycan (SLRP) family proteins play important roles in a number of biological events. Here, we demonstrate that the SLRP family member Asporin (ASPN) plays a crucial role in the early stages of eye development in Xenopus embryos. During embryogenesis, ASPN is broadly expressed in the neuroectoderm of the embryo. Overexpression of ASPN causes the induction of ectopic eyes. By contrast, blocking ASPN function with a morpholino oligonucleotide (ASPN-MO) inhibits eye formation, indicating that ASPN is an essential factor for eye development. Detailed molecular analyses revealed that ASPN interacts with insulin growth factor receptor (IGFR) and is essential for activating the IGF receptor-mediated intracellular signalling pathway. Moreover, ASPN perturbed the Wnt, BMP and Activin signalling pathways, suggesting that ASPN thereby creates a favourable environment in which the IGF signal can dominate. ASPN is thus a novel secreted molecule essential for eye induction through the coordination of multiple signalling pathways.

KEY WORDS: ASPN, Xenopus, Eye induction, Anterior-posterior polarity, IGF

INTRODUCTION

The visual system is conserved among a broad range of species from invertebrates to vertebrates, and is an important afferent component of the central nervous system transducing external stimuli from the periphery to the central part of the body (Kumar, 2001). The sensory organ of the visual system, the eye, comprises a number of different cell types, including neurons and glial cells, development of which is orchestrated in a complicated yet precise manner (Kohwi and Doe, 2013; Reese, 2011). How these cells differentiate and organise themselves has been a longstanding research interest, not least from a clinical point of view, as disorganisation of the eye structure from a functional and morphological point of view, can have a severe impact on quality of life. Unsurprisingly, a lot of research effort has been invested to generate retinal tissues in test tubes, mainly for transplantation purposes, but also out of a fundamental scientific interest. In vitro generation of retinal precursor cells is now technically feasible. In frogs, combining dorsal mesoderm and animal cap explants induces a fairly organised eye structure (Sedohara et al., 2003). In addition, animal cap cells that overexpress a cocktail of eye-inducers Chordin, Noggin and Follistatin (Harland, 2000; Muñoz-Sanjuán and Brivanlou, 2002; Ozair et al., 2013). These secreted factors are BMP antagonists, and induce a set of transcription factors including ET (eye T-box), Pax6 and Rx (Rax) in the broad area of the presumptive forebrain region (Zuber, 2010; Zuber et al., 2003). These transcription factors are required for the development of the whole forebrain (Klimova and Kozmik, 2014; Lagutin et al., 2003; Mathers et al., 1997). They subsequently drive the downstream transcription network formed by Six3, Lhx2 and Optx2 (Six6), and the expression starts to be confined to a specific area in the diencephalon during the neurulation process, which is later termed field-specific transcription factors is dynamic and the expression patterns change over time. Although a number of secreted factors, including FGF and Wnt, are known to be involved during this specification (Ikedo et al., 2005), more secreted factors must be involved in the fine-tuning of the eye-field-specific transcription factors’ expression patterns.

In addition to the neural inducers, the signals mediated by receptor tyrosine kinases (RTKs), mainly induced by insulin growth factors (IGFs) and fibroblast growth factors (FGFs), have also been shown to have important roles in the eye development as well as the head formation. The RTK signals activate extracellular regulated kinases (ERKs), and prime the degradation of Smad1 in concert with GSK3β (Puentealba et al., 2007; Kuroda et al., 2005; Pera et al., 2003). Therefore, the two signalling systems of BMP antagonists and RTK signals cooperate with each other and enable the embryos to acquire competence and differentiate into the eyes.

In vitro generation of retinal precursor cells is now technically feasible. In frogs, combining dorsal mesoderm and animal cap explants induces a fairly organised eye structure (Sedohara et al., 2003). In addition, animal cap cells that overexpress a cocktail of eye-field transcription factors acquire a competence for retinal cell fate and differentiate into a functional eye when transplanted back into an embryo (Viczian et al., 2009). In mouse embryonic stem cells (ESCs), timed treatment with different cytokines results in the induction of eye-field-specific transcription factors (Ikedo et al., 2005). Furthermore, three-dimensional formation of the retinal cell precursors is also achievable under a specific differentiation conditions (Eiraku et al., 2011; Gonzalez-Cordero et al., 2013). In contrast to these outstanding accomplishments in manipulating eye development, the molecular basis of gene regulation has not fully been elucidated.

The family of small leucine-rich repeat proteoglycan (SLRP) proteins comprises a number of extracellular matrix proteoglycans...
that have been shown to be involved in a number of biological events, including development, growth and cancer, in recent years (Dellett et al., 2012; Edwards, 2012; Schaefer and Schaefer, 2010). SLRPs feature leucine-rich repeat domains in the middle of their protein core, and can be categorised into five subclasses depending on their amino acid sequences (Dellett et al., 2012). Whereas most proteoglycans form large heterologous complexes of 300–400 kDa, SLRPs are thought to act mostly as monomers or possibly dimers of ~40 kDa (Goldoni et al., 2004; McEwan et al., 2006; Scott et al., 2004).

Once thought to be solely involved in collagen fibril organisation, SLRPs are now well known for their ability to modulate a number of intracellular signalling pathways (Chen and Birk, 2013; Wilda et al., 2004). This activity is exerted through interactions with specific extracellular signalling molecules and/or their receptor proteins. In *Xenopus* embryogenesis, SLRPs have been shown to play important roles in a number of developmental processes, such as germ layer specification, pattern formation and morphogenesis (Dellett et al., 2012; Kuriyama et al., 2006; Moreno et al., 2005; Muñoz et al., 2006). For instance, Biglycan binds to BMP4 and changes the interaction between BMP4 and Chordin (Moreno et al., 2005). Another SLRP, Tsukushi (TSK, TSKU), also binds to BMP, Wnt, Notch and inhibits their respective signalling pathways (Kuriyama et al., 2006; Morris et al., 2007; Ohta et al., 2004). These findings exemplify the diverse functions of SLRPs and their ability to coordinate multiple signals in a context-dependent manner.

During a systematic screening of the functions of SLRPs by means of mRNA injection in *Xenopus* embryos, we found that ASPN [ASP; also known as periodontal ligament-associated protein-1 (PLAP-1)] had a strong activity to induce ectopic eyes. ASPN was originally isolated from mice (Henry et al., 2001; Lorenzo et al., 2001), where it is expressed in cartilage and bone at the mid-gestation periods. ASPN inhibits the TGFβ signalling pathway, and an aspartic acid repeat polymorphism of the ASPN protein has been linked to osteoarthritis in humans (Kizawa et al., 2005). However, the activity of ASPN in the early stages of embryogenesis is still elusive, and the striking eye phenotype in *Xenopus* prompted us to analyse the function of ASPN in detail. We found that ASPN is an essential gene for eye induction that works by potentiating the IGF signalling pathway. Moreover, we found that ASPN interacts with a number of major signalling molecules and modulates their activities. Our results suggest that ASPN acts as a modulator for a number of signal molecules, and thereby contributes to specification of the eye-forming region.

RESULTS

**Structure and expression of *Xenopus* Asporin**

During a systematic investigation of SLRP functions (supplementary material Table S1), we found one of the clones demonstrating strong ectopic eye formation activity upon forced expression (as described below), and became interested in its detailed molecular function. This clone encoded a polypeptide sequence similar to human ASPN belonging to the class I family of SLRPs (Dellett et al., 2012), and drawing a phylogenetic tree showed that the clone contained a *Xenopus* orthologue of ASPN (Fig. 1A) (Henry et al., 2001; Lorenzo et al., 2001).

Sequence alignment of ASPN in different species further revealed that *Xenopus* ASPN has some typically conserved characteristics. *Xenopus* ASPN has a signal peptide and a 13 amino acid stretch comprising aspartic acid and asparagine (Fig. 1B, red) in its amino-terminal region, which is how ASPN was named (Henry et al., 2001; Lorenzo et al., 2001). This stretch is followed by a cysteine cluster with the C-X₃-C-X-C-X₄₋₅-C pattern, which is conserved among the class I SLRPs ASPN, Biglycan and Decorin, where the second cysteine is replaced by arginine in *Xenopus* (Fig. 1B) (Dellett et al., 2012). The characteristic stretch of eight leucine-rich repeats was also found to be conserved among vertebrate species.

To address the expression profile of ASPN during embryogenesis, we performed RT-PCR from different stages of embryos. ASPN expression was already apparent in unfertilised eggs and the level of expression gradually increased during embryogenesis (Fig. 1C).

We next performed whole-mount *in situ* hybridisation to map its expression pattern throughout development. During neurula stages, ASPN was ubiquitously expressed, with a little more abundance at the neural plate (Fig. 1D), including the presumptive eye field, as shown by Pax6 expression (Fig. 1E). The expression became more evident around the presumptive eye field at the tailbud stage (Fig. 1F). At stage 35, strong expression can be observed in the whole head region (Fig. 1G).

In order to supplement our *in situ* data, we quantified the expression of ASPN in different areas of the embryos. We prepared explants of animal cap, dorsal marginal zone (DMZ) and ventral marginal zone (VMZ) and assayed for ASPN by qRT-PCR. Animal cap explants from embryos pre-injected with *Chordin* (*Chd, Chrd*) to mimic the forebrain (Fig. 1Hii) (Sasai et al., 1995) or *Chd* plus Wnt8 to mimic more posterior neural domains (Fig. 1Hiii) (Takai et al., 2010) showed enhanced ASPN expression compared with animal caps from uninjected control embryos (Fig. 1Hi). ASPN expression in explants from DMZ (Fig. 1Hiv) and VMZ (Fig. 1Hv), mimicking dorsal and ventral mesoderm, respectively, showed low ASPN expression similar to that seen in the control animal cap. *Otx2* (Chow et al., 1999) and *Krox20* (Egr-2) expression (Nieto et al., 1991) (supplementary material Fig. S1) confirmed the character of the explants.

Together, these observations revealed that ASPN is expressed during early embryogenesis, and prompted us to investigate its embryonic functions, especially in neural development.

**Overexpression of ASPN induces ectopic eyes**

During the initial screen of the SLRPs’ activities by overexpression, we found a striking ectopic eye-like structure upon microinjection of mRNA encoding ASPN (Fig. 2A,B) and we decided to analyse this structure in detail.

We microinjected ASPN mRNA into dorsal animal blastomeres, and examined the phenotype at stage 42 (tadpole stage; see Fig. 2C–F,G for control injection). Microinjection of 1 ng of mRNA induced enlarged eyes (Fig. 2D–F,G,I), whereas higher doses induced ectopic pigmented eye-like structures (Fig. 2E–H,I). In order to characterise the induced pigmented structure, we performed Haematoxylin and Eosin staining on the sectioned tadpoles. The pigmented epithelium was thicker in the enlarged eyes on the injected side, and a separate retinal layer structure was occasionally found (Fig. 2G). Additionally, in the case of the embryos that had the ectopic pigmented structure, the pigment was never found inside the ectopic structure and the induced structure had an epithelial character (Fig. 2H). Notably, we did not find any expansion of the cement gland (the anterior-most structure) suggesting that ASPN function is not entirely the same as IGF signalling (Pern et al., 2001), and it also differs from the effects of cerberus (Fig. 2C–E,G) (Bouwmeester et al., 1996). Based on these observations, we supposed that this condensed pigment structure formed ectopically was a pigmented epithelium of the
retina, and the retinal structure was induced by the forced expression of ASPN.

In order to verify this hypothesis, we performed immunohistochemistry with antibodies specific to the different cell types of the eye. As a result, β-Crystallin (lens; Fig. 2J,K) Glutamine Synthetase (Müller glia; Fig. 2L,M) and Hu-C/Hu-D (Elavl3/Elavl4; retinal ganglion and amacrine cells; Fig. 2N,O) expression was found in the ectopically induced tissue. SLRP family members often exhibit overlapping functions, as is the case with Tsukushi and Biglycan for the induction of the organiser (Moreno et al., 2005; Ohta et al., 2004). To examine whether other SLRP members induce a similar eye phenotype to ASPN, we overexpressed Lumican, Decorin, Epiphycan and Chondroadherin in the Xenopus embryos and analysed the resulting eye phenotype (Fig. 2P; see supplementary material Fig. S2A-C for representative images). Both Lumican and Epiphycan induced a weak expansion of the normal eye, whereas the other SLRPs had no effect on the embryos’ eyes.

Together, these data suggest that ASPN specifically induces an eye structure containing retina, retinal pigmented epithelium (RPE) and lens.

ASPN induces eye-field-specific transcription factors (EFTFs) both in vivo and in vitro

In order to address the earlier effects of ASPN overexpression, we analysed gene expression patterns by whole-mount in situ hybridisation. ASPN mRNA was injected into a single dorsal animal blastomere at the 4-cell stage and the embryos were cultured until the early tailbud stage. Expression of the EFTFs Rx1 (Rax) (Mathers et al., 1997) (Fig. 3A,B; 90%, n=11) and Pax6 (Chow et al., 1999) (Fig. 3C,D; 100%, n=11) was clearly expanded or appeared
ectopically in the ASPN-overexpressing side of the embryo, whereas the *Otx2* (Chow et al., 1999) expression pattern remained unchanged (Fig. 3E,F; 100%, *n* = 11) (Blitz and Cho, 1995). By contrast, the markers *FoxG1* (*XBF1*; telencephalon; Bourguignon et al., 1998) (Fig. 3G,H; 50%; *n* = 20), *En2* (midbrain-hindbrain junction; Hemmati-Brivanlou et al., 1990) (Fig. 3I,J; 81.5%, *n* = 27) and *Krox20* (hindbrain/rhombomere 3 and 5; Nieto et al., 1991) (Fig. 3K,L; 100%, *n* = 12) were downregulated. These observations suggest that ASPN specifically encourages retinal development in vivo.

We next investigated the function of ASPN in vitro. For this purpose, we prepared animal cap explants from embryos injected with the *ASPN* mRNA, and analysed their gene expression when the sibling whole embryos reached stage 22. We found that ASPN increased *Sox2* and *NCAM* (general neural), *XAG1* (*AG1*; cement gland), *FoxG1* (telencephalon), *Pax6* and *Rx1* (forebrain and eye regions) and *Otx2* (forebrain and midbrain), whereas *En2* (midbrain and hindbrain junction), *Krox20* (hindbrain), *Slug* (*Snai2*; neural crest) and *c-Actin* (*cardiac Actin*; mesoderm) were not induced by ASPN (Fig. 3M), suggesting that ASPN can induce eye development on its own.

Taken together, these data show that ASPN has an ability to induce eye formation both in vivo and in vitro.

**ASPN is essential for the eye development**

To understand better the role of ASPN in early embryogenesis and eye development, we performed loss-of-function analysis using morpholino oligonucleotides (MOs) of *ASPN* (supplementary material Fig. S3A).

The *ASPN* morpholino (*ASPN-MO1*) was injected into the dorsal animal blastomere at the 4-cell stage and the phenotype was categorised at stage 42. Embryonic eyes (J,L,N) and the pigmented structure induced by injection of *ASPN* (K,M,O) were analysed at stage 42 with *β*-Crystallin (J,K), Glutamine Synthetase (L,M) and Hu-C/Hu-D (N,O) antibodies. Green, immunohistochemical signal; blue, DAPI. (P) The phenotypes found following injection of SLRP family members. SLRP family members were injected at 3 ng into a dorsal animal blastomere at the 4-cell stage and the phenotypes were categorised at stage 42. Key is shown in L.
Finally, we attempted to unveil the relationship between the neural inducer Chordin and ASPN. For this purpose, we prepared animal caps injected with Chordin (Sasai et al., 1994) and found an elevation in forebrain gene expression (Fig. 4Y). By contrast, when ASPN-MO was combined with Chordin mRNA, the expression was severely downregulated (Fig. 4Y), suggesting that ASPN acts downstream of the neural inducer Chordin.

In summary, ASPN is required for eye development, especially during the initial stages of the whole developmental process.

**ASPN induces eye development mainly via IGF receptor-mediated signalling pathway**

The ability of ASPN to produce an ectopic eye was reminiscent of that of IGF (Pera et al., 2001; Richard-Parpaillon et al., 2002). In addition, a previous study has shown that some SLRPs bind to the IGF receptor (Schaefer and Iozzo, 2008). These facts prompted us to investigate how ASPN is associated with the IGF signalling pathway.

We first asked whether ASPN activates the same signalling pathway as IGF. Because IGF has been shown to induce phosphorylation of ERK and AKT both in cultured cells and in the animal cap (Rorick et al., 2007; Wu et al., 2006), we examined whether ASPN activates the same intracellular signalling molecules. For this purpose, we prepared conditioned media of secreted ASPN and IGF2 from HEK293 cells, and applied these media onto another set of HEK293 cells that had been cultured separately. The cells treated with either ASPN or IGF2 activated the phosphorylation of AKT and ERK within 20 min of the treatment (Fig. 5A, lanes 2,3), suggesting that ASPN and IGF share the same downstream intracellular signalling pathways.

Next, we investigated whether ASPN forms a complex with the IGF1 receptor, and performed an immunoprecipitation assay. We co-transfected HEK293 cells with plasmids encoding ASPN and IGF1 receptor (IGF1R) and analysed cell lysates 24 h post-transfection. We found that ASPN does indeed establish a complex with IGF1R (Fig. 5B).

To confirm that the ASPN signal is transduced via IGF1R, we injected the dominant-negative version of the IGF1 receptor (dnIGFR) (Pera et al., 2001) together with ASPN mRNA and observed the phenotype of the eyes at the tadpole stage (see Fig. 5C for control; n=20). In contrast to the ectopic eye formation following the single injection of ASPN mRNA (Fig. 5D; 12%, n=11), the combined injection of ASPN and dnIGFR significantly decreased the size of the eyes (Fig. 5E; 22.6%, n=62).

To establish further the relationship between ASPN and IGF, we conversely perturbed the function of ASPN with ASPN-MO. As reported by the previous study, IGF2 injection caused enlarged eyes (Fig. 5F; 90%, n=22) (Pera et al., 2001). This enlargement was, however, blocked by co-injection of ASPN-MO (Fig. 5G; 91%, n=23). Thus, eye development requires both ASPN and IGF signals.

In order to confirm the necessity of both ASPN and IGF at the molecular level, we conducted an animal cap assay. For this purpose, we co-injected ASPN and dnIGFR or IGF2 and ASPN-MO and assayed the expression of Pax6 and Rx2a (Rax). We found that the expression of both genes was downregulated by the inhibiting constructs (Fig. 5H, I), suggesting that both ASPN and IGF are required for the early steps of eye development.

We also attempted to identify the interacting point of ASPN and IGF signals. We prepared animal caps injected with dnIGFR or ASPN-MO and treated them with conditioned media containing secreted ASPN or IGF2, respectively. The phosphorylation of ERK, which was activated in the control explants, was inhibited.
by the injection of dnIGFR or ASPN-MO (supplementary material Fig. S4). These data further suggest that both signals of ASPN and IGF interact with each other at the initial steps of their signalling pathways, but not with a secondary effect interfering with the transcription of other genes.

Together, these data demonstrate that ASPN induces eye development by regulating the IGF signalling pathway through a physical association with the IGF1 receptor.

ASPN interacts with and antagonises Nodal, BMP and Wnt molecules

Eye induction is regulated by a number of signalling molecules (Ikeda et al., 2005). Because it has been shown that SLRP family members interact with and inhibit the function of a number of signalling compounds in a context-dependent manner (Dellett et al., 2012), we investigated how ASPN affects some of the important signalling pathways, such as the Nodal/Activin, BMP and Wnt pathways.

First, we asked how ASPN influences these other signalling pathways. For this purpose, we injected reporter constructs of the Activin response element (ARE; for Nodal/Activin), BMP response element (BRE; for BMP signals) or TOPFLASH (for Wnt), together with mRNAs encoding Xnr1 (for ARE), BMP4 (for BRE) or Wnt8 (for TOPFLASH) in the embryos, and confirmed that all reporter activities were elevated at early gastrula stage. By contrast, when ASPN was co-injected with any of these signalling molecules, their activity was reduced significantly (Fig. 6A).

In order to confirm that ASPN has indeed the ability to inhibit these signals, we performed expression analyses, using either whole embryos or animal cap extracts. The animal caps were injected with ASPN mRNA and then treated with Nodal for 2 h. The expression of the Nodal target gene Mix.2 (Mixl1; which is upregulated following treatment with Nodal) was found to be downregulated in the ASPN-injected animal caps (Fig. 6B). Consistently, the injection of ASPN mRNA at the equator region at the 4-cell stage resulted in a reduction of Xbra (Brachyury, T) expression (Fig. 6C,D) at the gastrula stage, suggesting that mesoderm determination was severely influenced by ASPN. Furthermore, a shortened body axis phenotype was exhibited at the tadpole stage (supplementary material Fig. S6).

Next, we investigated inhibition of the BMP signal by ASPN more closely. We injected either Chordin, which is a well-known BMP inhibitor (Sasai et al., 1995), or ASPN mRNA and analysed the expression of general neural markers at the early neurula stage.
We found Sox2 and NCAM to be expressed at stage 14 in both conditions, which is consistent with the idea that ASPN inhibits the BMP signalling pathway (Fig. 6E).

We further investigated the involvement of ASPN in the Wnt signalling pathway. Again, we injected Wnt8 mRNA either on its own or together with ASPN mRNA and used qRT-PCR to analyse the expression of Xnr3 (Nodal3.1), which is one of the target genes of the Wnt signalling pathway (Yang-Snyder et al., 1996). We found that the expression of Xnr3 was induced when only Wnt8 was injected; however, Xnr3 levels were reduced by co-injection with ASPN (Fig. 6F).

In summary, these analyses revealed that ASPN is a multiple inhibitor for Nodal, BMP and Wnt signals.

We further attempted to find out how ASPN exerts its inhibitory effect on the aforementioned signalling molecules. As ASPN is a secreted factor, we hypothesised that ASPN forms complexes with the other signalling molecules. To verify this, we performed a binding assay: tagged versions of expression constructs encoding Xnr1 (Nodal1), BMP4 or Wnt8 were transfected into HEK293 culture cells together with a tagged version of ASPN, and a co-immunoprecipitation analysis was performed. Our results show that ASPN does indeed form complexes with all three tested molecules BMP4 (Fig. 6G), Xnr1 (Fig. 6H) and Wnt8 (Fig. 6I), suggesting that ASPN interacts with these molecules in the extracellular space and thereby impedes their activities.

Together, these data suggest that ASPN interacts with major signalling molecules that antagonise the eye formation in the extracellular space and blocks those activities (Fig. 6J).

**DISCUSSION**

**ASPN is a unique small leucine-rich repeat proteoglycan involved in eye development**

SLRPs have been shown to play significant roles in a number of biological events, including development, growth and the prevention of tumours (Brezillon et al., 2009; Dellett et al., 2012; Dupuis and Kern, 2014; Iozzo and Schaefer, 2010; Nikitovic et al., 2008; Shimizu-Hirota et al., 2004). Based on the amino acid sequence, ASPN is categorised with Decorin and Biglycan as a class I SLRP (Fig. 1A). Although all three members share leucine-rich repeats (LRRs), ASPN differs from Decorin and Biglycan in the number and spacing of cysteine residues at its N- and C-terminals (Henry et al., 2001; Lorenzo et al., 2001). ASPN can bind type I Collagen and competes with Decorin (but not Biglycan) for the collagen-binding site (Kalamajski et al., 2009). Furthermore, ASPN has been shown to bind directly to TGFβ and BMP2 and prevents them from binding to their respective receptors (Nakajima et al., 2007; Tomoeda et al., 2008; Yamada et al., 2007), which is in good agreement with our findings (Fig. 6). By contrast, Decorin interacts with the TGFβ1 and EGF receptors, and either enhances or diminishes their signal intensities (Iozzo and Schaefer, 2010). Likewise, Biglycan binds to BMP4 and regulates early embryogenesis or osteoblast differentiation (Chen et al., 2004; Moreno et al., 2005). So although ASPN, Decorin and Biglycan share similarities in terms of structure and amino acid sequences (Fig. 1A), the biochemical characteristics of these three proteins differ from each other. This functional diversity is probably...
caused by differences in binding partners and/or the affinities of the interactions.

This diversity is reflected in the embryonic activities of each protein. ASPN induces a strong eye phenotype, which was not observed after injection of the other class I SLRPs in *Xenopus* embryos (Fig. 1A and Fig. 2I; supplementary material Fig. S2A-C) (Kalamajski et al., 2009; Kizawa et al., 2005). In addition, SLRP members of other classes did not elicit an eye phenotype when they were overexpressed, the exceptions being the class I SLRP Lumican and class III SLRP Epiphycan, which occasionally induced a subtle eye phenotype (Fig. 2P; supplementary material Fig. S2B) (Kuriyama et al., 2006). This is consistent with the fact that the levels of ERK and AKT activation by Lumican and Decorin are different from each other (supplementary material Fig. S2D). Therefore, each SLRP seems to have its own unique functions and they are not redundant with each other.

ASPN was first identified as a TGFβ modulating molecule and was recognised as a causal gene for osteoarthritis (Henry et al., 2001; Kizawa et al., 2005; Lorenzo et al., 2001). In the mouse, ASPN is strongly expressed in the developing cartilage and its related tissues from the mid gestation period. In the mouse eye, ASPN expression was found in the sclera from 15.5 days post coitum, but significant expression has not been reported at earlier stages (Henry et al., 2001). As mouse models with disrupted *Aspn* gene expression have not yet been reported, it is currently unknown whether ASPN is involved in the early stages of eye development in mice. Therefore, the functions of ASPN in different organisms are of interest for future studies.

### ASPN antagonises BMP, Nodal and Wnt molecules and makes the IGF-mediated signal prominent

Our experiments have demonstrated that ASPN interacts with IGF and IGF1R, and activates the downstream signalling pathways (Fig. 5A; supplementary material Fig. S4 and Fig. S5A). In addition, both IGF and ASPN are required for the activation of these pathways (supplementary material Fig. S4). Furthermore, it is interesting to note that *ASPN* expression is induced by the neural inducer Chordin, but not by IGF (supplementary material Fig. S1), suggesting that there are two independent regulatory pathways of Chordin/ASPN and IGF for the induction of eye development. Taken together, one possible model is that the specification of the presumptive eye region relies on time and space, i.e. when and where the two molecules’ signals intersect with each other. However, according to the previous and present in situ hybridisation analyses, IGFs and ASPN are expressed ubiquitously (Fig. 1D) (Pera et al., 2001). Detection of the IGFs, ASPN and activated forms of ERK and AKT proteins at stage 10.5. (F-I) ASPN has neural-inducing activity. Animal caps injected with 500 pg Chd (lane 3) or 1 ng ASPN (lane 4) mRNAs were analysed at stage 14 by semi-quantitative PCR. (F) ASPN inhibits the Wnt signalling pathway. Animal caps injected with Wnt8 and ASPN mRNAs were prepared and the expression of Xnr3 was analysed at stage 10.5. (G-I) ASPN forms complexes with BMP4 (G), Xnr1 (H) and Wnt8 (I) proteins. In order to avoid artificial interactions in the same cells, each expression construct was separately transfected into HEK292 cells and cells were combined on the following day as indicated. The cell lysates were collected after two additional days and immunoprecipitation (IP) was performed with the HA antibody and western blotting (IB) was performed with the FLAG (G) or myc (H,I) antibodies (*P<0.01; **P<0.05; Student’s t-test). Error bars represent s.e.m.
Therefore, both the inducing activities and cellular responses seem to contribute to the spatiotemporal specification of the eye region.

Our experiments have further suggested that ASPN interacts with many other regulatory molecules, including BMP, Wnt and Nodal (Fig. 6G-I) in addition to IGF and IGFIR (Fig. 5A,B; supplementary material Fig. 5S/A), but has no, or very little if any, binding affinity to other receptor proteins (supplementary material Fig. 5S-B-D). This means that ASPN is not just an extracellular matrix protein that randomly binds to proteins, but rather is an active modulator for other signal molecules. Furthermore, ASPN can apparently regulate bound proteins in different ways. BMP, Wnt and Nodal are opposed by ASPN whereas the IGF signal is encouraged. This finding may account for the differences in the phenotypes by the overexpression of IGF and ASPN; ASPN exhibits a strong phenotype specifically in the eye (Fig. 2), whereas IGF induces the whole head structure including the cement gland (Pera et al., 2001).

It is also possible that ASPN binds additional, currently unidentified molecules. Actually, the expression of FoxG1 was decreased upon ASPN overexpression in the whole embryos (Fig. 3H), whereas the same overexpression instead upregulated FoxG1 expression in the simpler system of the animal caps (Fig. 3M), and this takes place probably due to differences in the proteins ASPN may bind to. The question of how ASPN interacts with many proteins is an intriguing research subject and deserves detailed quantitative (e.g. measuring dissociation constants) and systematic (e.g. high-throughput searching for interacting proteins) analyses.

There have been a number of intracellular molecules isolated as the ‘eye-maker’ factors (Rorick et al., 2007; Xu et al., 2012; Yang et al., 2003). Nevertheless, one advantage in identifying ASPN is that it is an extracellular protein and is easily applied to differentiating cells. We envisage that ASPN will be useful in improving the efficiency of eye production from embryonic stem cells. The increase in efficiency will be useful not only for clinical applications but also for the development of drug screening systems, and will consequently reduce the number of animals used to explore new therapeutic methods for retinal diseases. In conclusion, the discovery of ASPN raises possibilities of novel scientific and clinical applications.

MATERIALS AND METHODS
Isolation of ASPN
IMAGE (Integrated Molecular Analysis of Genomes and their Expression; http://www.imageconsortium.org) clones were purchased from Source Biosciences and the synthesised mRNA of each clone was injected into Xenopus embryos. The clone #6931202, encoding Xenopus asporin (NCBI Gene ID 495030), demonstrated a strong activity and we started further analyses. The very similar gene asporin-b, which exists probably because of the pseudotetraploidy of Xenopus laevis, was isolated in this study and has been registered in GenBank (accession number LC056842). Unless mentioned, all injection experiments were carried out with the mRNA of asporin-a (ASPN-a). The other genes that were screened are presented in supplementary material Table S1.

Embryonic manipulation, in situ hybridisation and immunohistochemistry
All animals in this study were subject to local and national ethical approval and guidance [University College London Ethical Committee, Cambridge University Ethical Committee and the Animals (Scientific Procedures) Act 1986 (UK Home Office)]. Frogs were purchased from Nasco (Salida, California, USA) and primed one week before use with 50 IU of pregnant mare’s serum gonadotropin (PMSG, Intervet) and then injected with 300-400 U human chorionic gonadotrophin (Intervet) on the evening before in vitro fertilisation was performed. Injection of mRNAs was performed with a fine glass capillary with a pressure injector (Harvard Apparatus).

Staging of the embryos was performed according to the normal table by Nieuwkoop and Faber (1967).

Embryos were harvested at the indicated stages, fixed with MEMFA (0.1 M MOPS [3-(N-morpholino)propanesulfonic acid (pH 7.4)], 2 mM EGTA (ethylene glycol tetraacetic acid), 1 mM MgSO4 3.7% formaldehyde) for 1h and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) if necessary. Antisense digoxigenin (DIG) RNA probes were synthesised with RNA polymerase (Roche). In situ hybridisation was performed as described elsewhere (Harland, 1991). Hybridisation buffer contained 1.3× saline-sodium citrate (SSC) pH 5.0, 1 mg/ml Torula RNA (Sigma), 0.2% Tween 20 detergent (Sigma), 0.5% CHAPS detergent (Sigma), 100 µg/ml heparin sodium salt and 50% formamide, and the hybridisation was performed at 60°C. Signals were visualised using BM Purple (Roche).

Three morpholino antisense oligonucleotides against ASPN were designed around the translation initiation site of the ASPN gene with the sequences of ATTTCTTCATGGTGTGTTCAGAG for ASPN-MO1, TATAAATTCTGCGGACATCATAGATAAA for ASPNa-MO2 and TCTCTTTAATGTTAATCCTACCTCTGA for ASPNb-MO2 (the underlined sequence is complementary to the translation initiation site; Gene Tools). ASPNa-MO2 and ASPNb-MO2 were injected together (termed ASPN-MO2) to ensure both alleles of ASPN genes were knocked down. The control oligonucleotide (control-MO; CCTCTTACCTCAGTTACAATTTATA) was used as a specificity control. For the rescue experiment, the mRNA of ASPNctv was used, which only contains the coding region of ASPN and is therefore unlikely to bind to either morpholino oligonucleotide.

For immunohistochemistry of sectioned eyes (Fig. 2), embryos fixed with 4% paraformaldehyde were incubated in 30% sucrose in PBS overnight. Embryos were then embedded in optimal cutting temperature (OCT) compound (Tissue-Tek) and sectioned at 10 µm thickness (Leica). The antibodies used in this study were: Glutamine Synthetase (Millipore, MAB302), Calbindin D-28K (Sigma, C-2724), Hu-C/Hu-D (Life Technologies, A21271), β-Crystallin (Abcam, ab90379). Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit antibodies (Abcam, A-11001 and A-11008) were used as secondary antibodies.

Animal cap, semi-quantitative and quantitative RT-PCR
Twenty animal caps for each condition were prepared at stage 10 and were cultured in Steinberg’s solution [58 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO3)2, 0.83 mM MgSO4, 4.6 mM Tris-HCl (pH 7.4)] (Sive et al., 2000) until the indicated time points and RNA was extracted with an RNeasy RNA extraction kit (Qiagen). Complementary DNAs (cDNAs) were synthesised with the reverse transcription by Superscript II with random hexamers (Life Technologies). Semi-quantitative RT-PCR was performed with the Platinum Taq DNA polymerase (Life Technologies). Primer sequences were taken from previous reports (Mizuseki et al., 1998; Shimizu et al., 2013) and the De Robertis laboratory webpage (http://www.hhmi.ucla.edu/derobertis/protocol_page/Pdfs/Frog%20protocols/Primers%20for20RT-PCR.pdf). Quantitative RT-PCR (qRT-PCR) was performed with the 7900 HT Fast Real-Time PCR machine (Applied Biosystems) with the SYBR Green detection system (Applied Biosystems). Each gene expression level was normalised to that of ODC (ornithine decarboxylase). Primer sequences for qRT-PCR are available in supplementary material Table S2.

Transfection, immunoprecipitation and western blotting
Human embryonic kidney HEK293 cells (ATCC number CRL-1753) were maintained with Dulbecco’s Modified Eagle’s medium (DMEM; Gibco) supplemented with 10% foetal bovine serum (Gibco) and antibiotics (penicillin and streptomycin) (Life Technologies). For preparing the conditioned media, HEK293 cells were transfected with the expression vector pCMV carrying the indicated gene and were incubated for three days in Opti-MEM (Life Technologies). The cells that had separately been transfected in Medium (DMEM) and conditioned media.

Cell extracts were prepared in TN Buffer [150 mM NaCl, 5 mM KCl, 0.5% NP-40 detergent, 10 mM Tris-HCl (pH 7.8)] with protease inhibitor cocktail (Roche). For the analysis of phosphorylated proteins, 5 mM NaF...
and 1 mM Na2VO3 were supplemented to inhibit dephosphorylation. Immunoprecipitation was performed using Protein G sepharose (GE Healthcare) with the indicated antibodies. After overnight incubation, beads were washed three times with TN Buffer and analysed by western blotting as described previously (Wang et al., 2013). The antibodies to phosphorylated ERK (9101), ERK (9102), phosphorylated AKT (Ser473; 9272), and pERK (9102) were used. Horseradish peroxidase (HRP)-conjugated mouse or rabbit IgG (GE Healthcare) were used as secondary antibodies and signals were detected with ECL Western Blotting Detection Reagents (GE Healthcare).

**Reporter assay**

The reporter constructs of ARE-luc (Activin-Responsive Element; Chen et al., 1997), TOPFLASH (the TCF/LEF Optimal Promoter monitoring the WNT activity; Upstate) and BRE-luc (BMP responsive element; Tozer et al., 2013) were used. pRL-CMV (Promega) was used as a normalisation control, and luciferase assays were performed using a dual-luciferase assay system (Promega).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

S.-O. conceived the project; K.L., M.K.-Y. and T.B. carried out the phenotypic analysis; N.S., H.D. and H.H. performed the mechanistic analysis; all authors analysed the data; N.S., K.L. and S.-I.O. wrote the manuscript.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/doi/10.1242/dev.124438/DC1

**References**


Figure S1. Expression of ASPN and other related genes in various explants. Expression levels of ASPN (A), Otx2 (B) and Krox20 (C) in various types of explants, as assayed by qRT-PCR. Animal caps (control (i) or injected with mRNAs of 500 pg IGF2 (ii), 500 pg Chd (iii) or 500pg Chd + 100 pg Wnt8 (iv)) and dorsal marginal zone (DMZ; v) and ventral marginal zone (VMZ; vi) were prepared at stage 10.5 and assayed at stage 18. Note that the data indicated with (†) are identical to those in Fig.1H.
Figure S2. Characterisation of Lumican and Decorin. (A-C) Representative images of the embryos injected with 3 ng ASPN (A), 3 ng Lumican (B) and 3 ng Decorin (C) mRNAs. (D) Differential activation of ERK and AKT by SLRP proteins. Control (i), ASPN-myc (ii), Lumican-myc (iii) or Decorin-myc (iv) expression media were prepared and applied onto HEK293 cells as in Fig. 5A.
Figure S3. Designation of morpholino oligonucleotides against ASPN and the phenotypes caused by the ASPN-MO2. (A) In addition to ASPNa, which this study is based on, we found another genome sequence probably due to the pseudotetraploidity, and termed it ASPNb. The nucleotide sequences (black characters) around the start codon (circled) of Xenopus ASPN and the sequences of ASPN-MO1 (red) ASPNa-MO2 (blue) and ASPNb-MO2 (purple) are shown. (B-D) Representative images from the injection of 20 ng control-MO (B), 20 ng ASPN-MO2 (C) and 20 ng ASPN-MO2 together with 1 ng of the coding region of ASPN (ASPN_{CDR}) mRNA (D). (E) Quantification of the phenotypes. For the rescue experiment embryos were injected with either 20 ng ASPN-MO2 and 1 ng ASPN_{CDR}, or 20 ng ASPN-MO2 and 3 ng ASPN_{CDR} and the phenotypes analysed at stage 41.
Figure S4. Both IGF and ASPN are required for the full activation of ERK. Animal cap explants were prepared from 3 ng control β-Galactosidase (i,ii,iv), 3 ng dnIGFR mRNA (iii), 20 ng control-MO (v) or 20 ng ASPN-MO (vi) injected embryos and were incubated with the conditioned media expressing control (i,iv), ASPN (ii,iii) or IGF2 (v,vi) for 20 minutes. The explants were analysed by western blotting using phosphor-ERK or ERK antibodies.
Figure S5 Interactions between ASPN and other molecules. The expression plasmids encoding ASPN-HA, IGF2-myc (A), Activin receptor (ActR)-FLAG (B), BMP receptor (BMPR)-FLAG (C) and Fzd4-CRD (the cysteine-rich domain in the extracellular part of Frz4)-myc-FLAG (D) were transfected as in Fig. 6G-I. The cell extracts were analysed by coimmunoprecipitation assays.
Figure S6. The phenotypes caused by the ventral injection of *ASPN* mRNA. 3 ng of *ASPN* mRNA was injected at the equator regions of one of the blastomeres at 4-cell stage and the phenotype observed at stage 42. In contrast to the control embryos (A), the injected embryos exhibited shortened bodies (B).

Table S1. The SLRPs used for the screening.

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Table S2. The primer sequences for semi-quantitative PCR and qRT-PCR.

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