INTRODUCTION

Development of the vertebrate eye depends on the coordinated interaction of three distinct tissues: neuroepithelium, overlying surface ectoderm and mesenchyme. In one of the earliest steps of eye formation, anterior neuroepithelium of the neural tube gives rise to the optic vesicles (OVs) by evaginating bilaterally (Fig. 1). Each OV extends until it contacts the surface ectoderm (SE). After contact, the distal portion of the OV invaginates thus initiating formation of the bilayered optic cup. The inner layer of the optic cup forms the multiple layers of the neural retina (NR), while the outer layer remains a simple cuboidal epithelium, which differentiates as retinal pigmented epithelium (RPE). Simultaneously, the SE, at its point of contact with the OV thickens into a placode that will first form a vesicle and later differentiate into the lens.

Although different in form and function, the NR and RPE derive from common progenitors in the OV. Different experimental approaches have demonstrated that single cells of the OV are bipotential, able to form both NR and RPE (Mikami, 1939; Dragomirov, 1937; Stroeva, 1960; Dorris, 1938; Lopashov, 1963). Thus, it has been postulated that patterning or segregation of the OV into separate NR and RPE domains must arise from local inductive events (Mikami, 1939; Detwiler and VanDyke, 1954; Dragomirov, 1937). One hypothesis states that tight apposition between the distal tip and the SE may be necessary for exclusion of RPE-inducing mesenchymal cells (Lopashov and Stroeva, 1961). However, it is also possible that SE or SE plus mesenchyme exerts a direct instructive role, specifying tissue-specific domains in the OV. Alternatively, information defining these tissue patterns could be transmitted by local contact between inner and outer layers of the optic cup, thereby establishing the NR; when contact between these two layers is prevented, the inner prospective NR differentiate as RPE (Orts-Llorca and Genis-Galvez, 1960).

SUMMARY

Patterning of the bipotential retinal primordia (the optic vesicles) into neural retina and retinal pigmented epithelium depends on its interaction with overlaying surface ectoderm. The surface ectoderm expresses FGFs and the optic vesicles express FGF receptors. Previous FGF-expression data and in vitro analyses support the hypothesis that FGF signaling plays a significant role in patterning the optic vesicle. To test this hypothesis in vivo we removed surface ectoderm, a rich source of FGFs. This ablation generated retinas in which neural and pigmented cell phenotypes were co-mingled. Two in vivo protocols were used to replace FGF secretion by surface ectoderm: (1) implantation of FGF-secreting fibroblasts, and (2) injection of replication-incompetent FGF retroviral expression vectors. The retinas in such embryos exhibited segregated neural and pigmented epithelial domains. The neural retina domains were always close to a source of FGF secretion. These results indicate that, in the absence of surface ectoderm, cells of the optic vesicles display both neural and pigmented retinal phenotypes, and that positional cues provided by FGF organize the bipotential optic vesicle into specific neural retina and pigmented epithelium domains. We conclude that FGF can mimic one of the earliest functions of surface ectoderm during eye development, namely the demarcation of neural retina from pigmented epithelium.

Key words: Fgf, Neural retina, Optic vesicle, Eye development, Retrovirus, Vertebrate

FGF1 patterns the optic vesicle by directing the placement of the neural retina domain

Jeanette Hyer, Tatsuo Mima and Takashi Mikawa*

Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, USA

*Author for correspondence (e-mail: tmikaw@mail.med.cornell.edu)

Accepted 18 December 1997: published on WWW 4 February 1998
Pittack et al., 1997). A cognate receptor for these FGFs, FGFR1, is found throughout the OV (Wanaka et al., 1991). FGFR2, a second potential receptor, colocalizes with FGFR1 in the early optic cup (Tcheng et al., 1994).

A number of studies have addressed the role of FGF signaling in retinal development. In the differentiation of the NR, FGF1 has been shown to elevate cellular opsin levels (Hicks and Courtois, 1988) and FGF2 increases the number of opsin-positive cells (Hicks and Courtois, 1992). Significantly, FGFs are able to transform the differentiated RPE into NR (Guillemot and Cepko, 1992; Park and Hollenberg, 1991). These data have led to the suggestion that FGF might be the true inducer of the NR. However, when FGF2 is removed from the SE, the OV is still patterned into NR and RPE domains (Pittack et al., 1997); thus, another component(s) of the SE may be responsible for OV patterning. Previous work on the commitment and differentiation of the NR lineage has been based on in vitro assays or on transdifferentiation of RPE after optic cup stages of development. There have been no experiments focusing on early steps of OV development in vivo. In the present study, an in vivo assay has been established for examining the influence of FGF on patterning of the OV. Evidence is presented that, in the absence of SE, optic vesicle cells can initiate the differentiation program into both neural retina and pigmented epithelium, and that FGF alone, mimicking surface ectoderm, can drive the cells to organize into two distinct retinal domains.

MATERIALS AND METHODS

Virus constructs and production

Construction of the viral vectors and propagation of the recombinant viruses have been described elsewhere (Mima et al., 1995; Itoh et al., 1996). Biological activities of the FGF virus, the FGFR1 virus and the antisense FGFR virus have been used previously to assess the role of FGF signaling in cellular differentiation and proliferation within somitic mesoderm, limb mesenchyme, cardiogenic mesoderm and coronary vasculature as described in Itoh et al. (1996), Mima et al. (1995) and Mikawa (1995).

Microinjection

Fertile White Leghorn chicken eggs (Truslow Farms, NJ) were incubated at 38°C until Hamburger-Hamilton (HH) stage 10 (10 somites, ~1.5 days). A small hole was made in the egg shell and the extraembryonic membranes overlying the embryo removed. With a glass capillary, approximately 1 nl of virus solution, at a concentration of ~10^8 virions/ml, was pressure-injected using a picospritzer (General Valve Co., NJ) into the selected area. Eggs were sealed with Parafilm and reincubated until indicated stages.

Implantation

The surface ectoderm surrounding the optic vesicle was surgically removed at stage 10 using 1 µl of Nile blue sulfate (Sigma, 1.5% in water). As described in Yang and Niswander (1995), this causes a slight blistering of the ectoderm and facilitates the removal of SE without disturbing the underlying neuroectoderm. Upon removal, the SE was replaced by FGF-producing fibroblasts. D17 fibroblasts, previously infected with a retrovirus encoding FGF1 and β-galactosidase (β-gal), were plated on polyester membranes (Costar) cut to 0.45x0.70 mm. Membranes were placed onto the optic vesicle at various sites. The average embedded membrane held ~10^5 cells and 1-10 ng of FGF was secreted from 10^3 infected fibroblasts per day (Itoh et al., 1996), as assayed by immunoblot and dosage studies on PC12 neurite outgrowth (Togari et al., 1985). Eggs were sealed with Parafilm and reincubated until the indicated stage.

Immunohistochemistry

Embryos were removed from eggs, fixed overnight at 4°C in 2% paraformaldehyde, stained with X-gal at 37°C overnight and then processed for cryo- or paraffin sectioning. HU mouse monoclonal antibody (Kostyk et al., 1996; Marusich et al., 1993), a generous gift from Dr Henri Furneaux (SK1, New York) and Dr Michael Marusich (Univ. of Oregon, OR) was used on cryosections at a dilution of 1:500. Anti-β-galactosidase rabbit polyclonal antibody (Cappel) was used on 10 µm cryosections at a dilution of 1:500. Secondary anti-mouse and anti-rabbit antibodies conjugated to indicated fluorochromes (American Qualex) were used at a dilution of 1:500. RA4 mouse monoclonal antibody (McLoon and Barnes, 1989), a generous gift from Dr Steven McLoon (Univ. of Minneapolis, MN) was used on 10 μm paraffin section at a dilution of 1:500, together with goat anti-mouse secondary conjugated to alkaline phosphatase (Sigma) and BCIP/NBT (Calbiochem) as chromogen.

RESULTS

The NR and PE are patterned prior to optic cup formation

In vitro, RPE is competent to transdifferentiate into NR tissue in response to FGF. Paradoxically, shortly after optic cup formation, the NR itself begins to secrete FGFs in vivo, yet the RPE continues to develop as it would in the absence of FGFs. These results lead to the hypothesis that, in the optic cup, the juxtaposed NR inhibits the RPE from adopting a NR cell fate, implying that NR and RPE domains are established in the optic cup stage of development. Alternatively, in the optic cup, RPE might not see or respond to FGF secreted from the juxtaposed NR. We asked whether the RPE is capable of forming NR in the context of an intact optic cup by introducing a constitutive FGF signal into the forming optic cup. This was accomplished by using a replication-defective retrovirus to express FGF ligand or the FGFR gene with a reporter β-gal. To control for non-specific effects of viral infection, a vector expressing only β-gal was used in parallel experiments.

Viruses were microinjected into the presumptive RPE zone of the OV at E1.5 (HH stage 9) and the eyes examined at E7 (Fig. 2A). Staining with X-gal identified the infected cells (Fig. 2B,C). Infection with β-gal virus (Fig. 2B) gave rise to blue sectors of X-gal staining in the eye; FGF1-producing virus, which produces ectopic sources of FGF within the RPE, gave rise to eyes with sectors lacking pigmentation around sites of viral infection (Fig. 2C). β-gal virus had no apparent effect on the fate of these cells; sections showed that, when the presumptive RPE was infected, pigmented cells were unchanged in number or distribution (Fig.
Retroviral infection of the presumptive NR with the β-gal vector also had no apparent effect (Fig. 2E) on neuronal numbers or distribution. Sections through FGF-infected eyes, however, revealed that zones lacking pigment (Fig. 2C) consisted of a double-layered NR (NR and iNR) as evidenced by localization of Hu protein (Fig. 2F), an RNA-binding protein expressed in neurogenic cells prior to overt differentiation. Hu protein was never seen in the RPE or in any non-neuronal tissues (Fig. 2D), making it an ideal marker for identifying cells of the neuronal lineage within the developing retina.

Infection of presumptive NR with the FGF-producing virus did not induce the adjacent RPE to differentiate into NR (data not shown). In contrast, infection of the mesoderm underlying the presumptive RPE domain with FGF virus transformed the RPE into NR-expressing Hu (Fig. 2G). Infection of the RPE with FGFR-encoding virus did not alter the developmental fate of the RPE and the tissue was identical to control tissue infected with the β-gal vector (data not shown). Using RA4 as a marker of differentiated cell types, such as ganglion cell bodies and axonal processes, we examined the induced NR. RA4 immunoreactivity was strongly positive in the induced NR (Fig. 2H), but was reduced in the same tissue when in close proximity to the RPE (Fig. 2I).

We conclude that the RPE is competent to form NR in the presence of endogenous NR during normal development. However, the RPE does not respond to FGF present in the NR even when supplied with high levels of receptor. These data do not support the hypothesis that inhibition of NR differentiation in the RPE is caused by endogenous NR since RPE can be transformed into NR when FGF is supplied from the underlying mesoderm. Since this second source of FGF could be mimicking the SE, we hypothesized that FGF coming from the SE might direct the formation of the NR. This implies that, by the optic cup stage of development...
development, delineation of NR and RPE domains have already been established.

**The surface ectoderm is required for optic vesicle patterning**

To test the hypothesis that SE determines the location of the NR, we removed the SE at E1.5, before it makes contact with the OV (Fig. 3A). The resulting embryos were examined at E4, E5 and E7. Examination in whole mount of the operated embryos revealed that the effects of ablation were restricted to eye development; overall facial and brain morphogenesis was indistinguishable from unoperated embryos (Fig. 3B,C). In the absence of the SE, the OV in most cases developed into a pigmented vesicle (86% of embryos, n=14). These remnants were severely reduced in size and had no lens, indicating that the SE had been successfully ablated. Sections through the eye remnant revealed that such eyes lacked the normal organization of RPE and NR tissues (Fig. 3D) and failed to form bilayered optic cups (Fig. 3E). Importantly, the cells of OV displayed an indeterminate phenotype. Retinal cells in these embryos were pigmented but, unlike the normal RPE, they were no longer arranged as a simple cuboidal epithelium. Instead, they formed

---

**Fig. 3.** Effect of ablating surface ectoderm on eye morphogenesis. (A) Whole-mount view of a day 7 embryo, showing overall brain and eye formation. (B) An embryo in which the surface ectoderm was removed at E1.5, showing dramatic and specific reduction of the eye (arrow). (C) Section through a normal optic cup of an E7 embryo, showing the correct relationship between columnar NR and epithelial RPE tissues. (D) Section through embryo treated as in B, showing a pigmented tissue in a columnar array (arrowheads, position of Hu-positive cells as in F). (E) Hu staining in a normal retina stains various cell types in the neural retinal layer (arrow); the RPE is not stained. (F) Hu staining of tissue section in D where Hu-positive cells (arrowheads) are present within the pigmented retinal layer. OT, the optic tectum; NR, the neural retina; PE, the retinal pigmented epithelium. Bars represent 1.0 mm.
the thickened retinal tissue typical of NR differentiation (Fig. 3D). Hu+ cells were sequestered within this pigmented and thickened retinal layer (Fig. 3F). Therefore, in the absence of the SE, the OV had lost patterning information but the capability to initiate the differentiation into NR and RPE was preserved.

**FGF organizes the optic vesicle into NR and RPE domains**

SE could be playing three major roles in this early stage of eye development: (1) regulating the proliferation of cells forming the retina, (2) inducing optic cup formation or (3) organizing RPE and NR domains. To determine what role FGF1 is playing, we introduced a new source of FGF1 ligand in the SE-ablated eye (Fig. 4A). FGF virus was injected into the mesoderm posterior to the OV, adjacent to presumptive RPE. The infected cells that co-expressed FGF1 and β-gal were identified by X-gal staining (data not shown). The resulting embryos were examined at E5.

An optic cup was never formed. The OV exhibited clearly delineated pigmented and non-pigmented domains, with the non-pigmented domains nearest the mesodermal source of exogenous FGF (55%, n=9) (Fig. 4B-D). In control experiments, where virus encoding only β-gal was injected, these effects were never seen. Sections through eye remnants in embryos infected with the FGF virus showed that the OV had not formed a bilayered optic cup but remained as a single-layered vesicle. β-gal protein was not found in NR tissue. The pigmented region had a cuboidal pigmented layer of cells, characteristic of RPE. The adjacent non-pigmented layer exhibited a columnar arrangement of cells typical of NR (Fig. 4E); at a distance from the transition point, strong Hu staining was observed (Fig. 4F), indicating that the unpigmented zone had been committed as NR. Hu staining was never robust in areas adjacent to pigmentation.

**FGF patterns through short-range interactions**

We removed the SE and placed an FGF signal at the distal and/or
medial positions of the OV (Fig. 5A). This procedure provided an FGF source that more closely mimicked the normal release of FGF ligand from SE by being spatially removed from the OV. It also eliminated the possibility that FGF-producing mesodermal cells might have been secreting unknown active factors consequent to infection. Fibroblasts co-expressing FGF1 and β-gal were plated onto polyester membranes and these implanted onto the exposed OV. Cells expressing only β-gal were used as controls for non-specific effects of implantation. Whole-mount X-gal staining of embryos revealed that β-gal-positive cells remained localized to the site of implantation (data not shown). Control implants of cells expressing only β-gal did not rescue the disarray in eye formation that results after SE ablation (100% of embryos, n=8) (Fig. 5B). Implantation of FGF-producing cells at single sites on the exposed OVs gave rise to eyes that were again divided by a sharp boundary into pigmented and non-pigmented zones (41% of embryos, n=22) (Fig. 5C,D). In all cases, the non-pigmented zone was restricted to the site where FGF was supplied to the OV. Importantly, when cells were placed at two sites so that FGF was supplied to both anterior and posterior regions of the OV, all pigmentation was lost (59% of embryos, n=22) (Fig. 5E-G). In the expanded non-pigmented layer, RA4 immunoreactivity could be detected (Fig. 5H,I), indicating that the non-pigmented zone had differentiated as NR.

DISCUSSION

FGF1 organizes the NR domain in the OV

In vitro studies, using explant assays, have demonstrated that the single cells of the OV are poised to differentiate as RPE or

**Fig. 5.** Effect of exogenously supplied FGF on NR differentiation. (A) Diagram of operation; FGF-expressing cells are implanted at distal tip of naked optic vesicle. (B) In control embryos, the optic vesicle forms a uniformly pigmented vesicle. (C,D) Examples of treated embryos at E7, asterisk indicates the site of implanted cells. (E-G) Examples of embryos treated with two implants so that entire optic vesicle is converted to a NR cell fate. (H) RA4 staining of a normal optic cup, arrow indicates the direction of cell migration and asterisk indicates RA4 staining of cell bodies, lumen is the vitreal cavity. (I) RA4 staining of sectioned eye remnant of G, arrow of cell migration points in opposite direction, since the NR did not form an optic cup, lumen is the enduring primary optic cavity, asterisk indicates RA4 staining of cell bodies. NR, the neural retina; PE, the retinal pigmented epithelium. Bars represent 0.2 mm.
NR and are not fixed at the early OV stage (Lopashov, 1963). During in vivo development, the OV is divided into RPE and NR domains at an early stage. Here we show that removing the SE causes a loss of the RPE/NR boundary. The OV becomes disorganized, and individual cells of the OV exhibit both pigmentation phenotype of the RPE and the tissue organization phenotype of the NR. This randomization of the OV is reminiscent of prior results with explants, which demonstrated that the OV can initiate differentiate into both NR and RPE without SE (Lopashov, 1963 p.11-25). Our results do not support the concept that SE is the inducer of the NR, rather they reinforce in vitro findings that all of the cells of the OV are bipotential and able to form both RPE and NR without instructive signals from the SE.

Although FGF2 has been demonstrated to induce a neuronal phenotype in cultured PC12 cells (Rydel and Greene, 1987), FGF2 has only been shown to influence the differentiation of selected cell types of the NR in the developing vertebrate eye (Zhao et al., 1996; Hicks and Courtois, 1992). A series of recent experiments support the notion that FGF is not an inducer of NR in vivo. McFarlane et al. (1996) transfected a dominant negative FGF receptor into the early eye field of the Xenopus embryo and found that the transgenic cells could fully differentiate. Pittack et al. (1997) applied FGF2-neutralizing antibodies to organ culture explants of embryonic chicken OVs and found that NR and RPE formed distinctly but that the NR did not express differentiated markers. The ability of the OV to spontaneously differentiate into NR tissue in explant cultures (Lopashov, 1963, p12) leads to the conclusion that, by the time the OV is formed, an inducer of the NR is not necessary. One view considers the process of induction during embryonic development to be the result of cumulative tissue interactions arising from several tissues (Jacobson, 1966). In the OV, induction of the NR by SE may be viewed as only the last step of a sequential series of inductions, one of which might involve FGF signaling. Although SE may produce other factors in addition to the FGFs that participate in patterning the OV, our data indicate that FGF alone substitutes for this organizational function of the SE.

In our experiments, two different approaches produced identical results. The OV is always organized such that the NR domain is closest to the FGF signal. When the SE is removed and the mesoderm underneath the OV produces FGF, the OV is organized such that the NR domain is oriented towards the FGF source in the mesoderm. RPE always formed on the other side of the OV away from the FGF-producing mesoderm. When the SE is removed and exogenous cells expressing FGF are placed on the OV, again the OV differentiates in an oriented fashion with NR closest to the FGF source. Implanting membranes carrying FGF-secreting fibroblasts enabled us to assess FGF responsiveness in different regions of the OV. We found no variations in FGF responsiveness between anterior, posterior or lateral sectors of the OV; the NR domain always developed nearest the source of the FGF. If two sources of FGF were applied to the OV, the RPE domain was lost completely and the entire OV differentiated as NR.

An interesting observation from the current study is that Hu and RA4, the markers of the NR, are always observed in areas separated from pigmentation and are not expressed near the NR/RPE boundary. The results suggest that critical NR cell numbers and/or NR-intrinsic factors are required for determination (Hu) and differentiation (RA4) of the NR. The patterns of Hu and RA4 expression supports the hypothesis that an intrinsic retinal growth factor is needed for the determination and differentiation of the NR (Altshuer et al., 1993; Watanabe and Raff, 1992; Sparrow et al., 1990; Reh, 1992), and might also indicate the presence of some inhibition of NR by PE in that transition zone. Such transition zone may correspond to the presumptive ciliary body.

**The cells of the OV respond to FGF1 in a short-range paracrine fashion**

In the above experiments, complete loss of RPE required two implanted FGF sources. This short-range FGF signaling fully recapitulated the expected action of the SE. Thus, the contact-dependent nature of NR patterning during morphogenesis insures that NR forms at the distal tip of the OV. Inductions prior to OV formation most likely endow the cells of the OV with the potential to form NR. FGF signaling reinforces that potential in a specific region of the OV, its distal tip. FGF from the SE is deposited in the extracellular matrix (Jenney et al., 1987), and limitations in diffusion of FGF ligand requires OV:SE contact to transmit requisite developmental cues.

The viral construct that we use to express FGF has a signal sequence derived from FGF4 upstream of the FGF1 gene (Forough et al., 1993). It is still uncertain how FGF1 and FGF2 are secreted from cells, as they lack signal peptides. When used to infect the tissue of the OV, the RPE cells respond to FGF in...
The OV sees FGF, a NR cell fate is chosen. As the NR has no FGF on gene transcription in the eye can be analyzed. This model can be tested since the downstream consequences of the OV, without reinforcement, slowly lose the potential to stabilize NR cell fates in that region of the OV. Proximal zones form NR (Fig. 6). As the distal OV contacts the SE, FGF first shows a clear down-regulation in the presumptive NR even at (Bovolenta et al., 1997) by the time optic cup forms. Otx-2 to the RPE [Otx-1 (Bonicello et al., 1993) and Otx-2 [Six-3 (Oliver et al., 1995), Pax-6 (Grindley, et al., 1995)] or prospective RPE. Other homeodomain proteins are expressed 2 (Nornes et al., 1990), all of which are absent from adjacent tissues are prevented from contacting one another by placement of an obstruction between the two cell populations, RPE developed into NR nearest the obstruction (Orts-Llorca et al., 1960), suggesting the presence of a local inhibitor.

Molecular mechanism of NR domain determination
Cells of the OV are not committed to a NR and PE fate early in development. Plasticity of these cells has been demonstrated well after optic cup formation with amphibian tissue. In chicken embryonic optic cups, plasticity of cell fate is seen up to embryonic day 3 (Dorris, 1938). Even after the NR becomes fixed in its fate, RPE cells remain plastic (Lopashov, 1963; Coulombre and Coulombre, 1965; Zhao et al., 1995).

In this present report, we show that the NR domain is reinforced at the distal tip of the OV by FGF signaling. One possible mechanism for specifying the NR domain is through the differential expression of genes between RPE and NR domains. FGF signaling might specify the NR domain by driving the expression of the specific transcription factors found in the OV, either inducing or inhibiting the expression of the appropriate factors in the NR. While still at the OV stage, the presumptive NR expresses Msx-2 (Monaghan et al., 1991), Chx-10 (Liu et al., 1994), Rx (Mathers et al., 1997) and Pax-2 (Nornes et al., 1990), all of which are absent from adjacent prospective RPE. Other homeodomain proteins are expressed throughout the OV and then become restricted to either NR [Six-3 (Oliver et al., 1995), Pax-6 (Grindley, et al., 1995)] or to the RPE [Otx-1 (Bonicello et al., 1993) and Otx-2 (Bovolenta et al., 1997)] by the time optic cup forms. Otx-2 shows a clear down-regulation in the presumptive NR even at the early OV stage. In one transdifferentiation assay, exogenous FGF2 in the media induces expression of Chx-10 from RPE tissue (Zhao et al., 1997).

We propose a model in which the entire OV is competent to form NR (Fig. 6). As the distal OV contacts the SE, FGF first stabilizes NR cell fates in that region of the OV. Proximal zones of the OV, without reinforcement, slowly lose the potential to realize a NR fate and eventually become “fixed” as RPE. This model can be tested since the downstream consequences of FGF on gene transcription in the eye can be analyzed. Consistent with this model is our demonstration that, wherever the OV sees FGF, a NR cell fate is chosen. As the NR has no significance to the organism unless it forms in proper relation to the rest of the eye, FGF signaling, by refining the NR-competent field, assures that the NR only differentiates at the distal tip of the OV.

The authors thank Henri Furenaux, Michael Marusich and Steven McLoon for their generous sharing of antibodies; D. A. Fischman, D. Herzlinger, J. Sparrow, A. Alvarez-Buylla and J. Kuhlman for their comments and discussions on this study and Ms L. Miroff, L. Ong and L.Cohen-Gould for their technical assistance. This work was supported in part by grants from the NIH, and the Mathers Foundation. T. M. is an Irma T. Hirschl Scholar. This work is in partial fulfillment of the doctoral degree of J. H. in the Graduate School of Medical Sciences of Cornell University.

REFERENCES


