FACTEURS IMPLIQUES DANS LA DIFFERENCIATION ET LA TRANSDIFFERENCIATION DE L'EPITHELIUM CORNEEN

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THESE

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FACTEURS IMPLIQUES DANS LA DIFFERENCIATION ET LA TRANSDIFFERENCIATION DE L'EPITHELIUM CORNEEN

FACTORS INVOLVED IN CORNEAL EPITHELIUM DIFFERENTIATION AND TRANSDIFFERENTIATION

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RESUME

Plusieurs questions concernant les mécanismes de différenciation de l'épithélium cornéen ont été abordées: premièrement, le cristallin est-il réellement l'inducteur de la cornée? La kératine 12 (K12) est-elle spécifique de l'épithélium cornéen ou bien est-elle exprimée aussi dans d'autres épithélia? Enfin, quels sont les rôles respectifs du gène Pax6, le chef d'orchestre de la morphogenèse oculaire et des messages qui pourraient être transmis par le stroma cornéen?

Chez l'embryon de poulet de 2/3 jours, Pax6 est exprimé dans les noyaux non seulement des futurs tissus oculaires, mais aussi dans le cerveau, ainsi que dans l'épithélium nasal et oral. Après l'individualisation de la cornée, Pax6 continue d'être exprimé tout au long de la vie non seulement embryonnaire, mais aussi de la vie adulte. Par contre, l'expression de Pax6 est éteinte après 7 jours d'incubation dans l'épithélium nasal et oral.

L'expression de K12, marqueur de différenciation de l'épithélium est facilement observable seulement à partir d'un stade embryonnaire relativement avancé : 14 jours d'incubation pour le poulet, 21 jours de gestation pour le lapin, et tout au long de la vie adulte. Cette expression est spécifique de l'épithélium cornéen.

J'ai transfecté le cDNA codant pour une forme active de Pax6 (couplée à l'activateur VP16) chez l'embryon de poulet de 2.5 à 3 jours d'incubation, par la technique d'électroporation in ovo. Le résultat est une orientation dorso/ventrale anormale de l'œil, montrant l'importance d'une régulation fine et précise de la quantité et de la localisation de la protéine Pax6. Cependant aucune formation ectopique de tissus oculaires n'en a résulté.

J'ai étudié le rôle du cristallin, présenté comme l'inducteur de la cornée. Contrairement à ce qui a été publié antérieurement, celui-ci est seulement requis pour la croissance de l'œil

mais ni pour la migration des fibroblastes formant le stroma de la cornée, ni pour l'expression de K12 dans son épithélium.

Afin d'étudier la question du rôle éventuel du stroma lors de l'activation des gènes Pax6, puis K12, j'ai réalisé plusieur types de recombinaisons épithélio/mésenchymateuses. Les recombinants ont été greffés sous la capsule du rein de souris athymique. Les expériences réalisées avec les tissus d'embryon de poulet montrent que Pax6 peut être éteint et le futur épithelium cornéen transformé en épiderme et en plumes seulement avant 5 jours d'incubation. En collaboration avec le Dr. David Pearton, nous avons montré que au contraire chez les mammifères, Pax6 peut être éteint et la formation d'un épiderme et de follicules pileux obtenus même à partir d'un épithélium cornéen prélevé chez l'adulte.

L'insuffisance du nombre de donneurs pour les greffes de cornée est un challenge. Nous nous sommes donc demandés si la transformation inverse de divers épithéliums en épithélium cornéen ètait réalisable. Ni l'association avec un stroma cornéen, ni la transfection de Pax6 n'a permis d'obtenir ce résultat.

Mots clé: poulet, cornée, interactions épithélio-mésenchymateuses, K12, cristallin, Pax6, lapin, transdifférenciation, peau.

SITUATION DU SUJET ET PRESENTATION DE LA THESE

Le développement de l'œil est un classique de l'embryologie. Au début du siecle dernier, Spemann (1901) avait le premier introduit la notion d'induction par des expériences restées célébres. Chez l'embryon d'amphibien, la greffe de la vésicule optique sous l'ectoderme de l'abdomen provoquait dans ce dernier la formation d'un cristallin. Ces expériences, qui ont déclanché toutes les recherches ultérieures en embryologie expérimentale, afin de déterminer les différentes interactions tissulaires responsables de la formation des divers organes…étaient entâchées d'erreur. Reproduites au début des années 90 à l'aide d'embryons donneurs marqués au FLDx elles montraient que dans les cas où un cristallin était obtenu, celui-ci était fluorescent, comme la vésicule optique. En fait la compétence de l'ectoderms à répondre à l'induction de la vésicule optique est restreinte à une partir de l'ectoderme céphalique (Grainger, 1992) et par une induction planaire issue de la plaque neurale.

En ce qui concerne la morphogénèse de la cornée, des expériences anciennes (review, Hay, 1980) d'excision de la vésicule cristallinienne chez l'embryon de poulet avaient conclu que cette dernière était responsable de l'induction de la cornée. Cette conclusion a depuis été reprise dans tous les livres d'enseignement de Biologie du Développement. Le groupe d'Overbeek à Houston a montré recemment que le cristallin est producteur de FGF10 (Govindarajan et al., 2000). Nous nous sommes alors demandé sile cristallin n'était pas simplement nécessaire à la croissance du globe optique. Nous disposions d'un anticorps monoclonal spécifique de la différenciation de l'épithélium cornéen pour analyser nos résultats.

La cornée est un organe épithélio-mésenchymateux. Elle est composée d'un épithélium pluristratié, non cornifié, qui provient de l'ectoderme embryonnaire et d'un tissu conjonctif à

l'ordonnancement très règulier, le stroma. Au cours du développement elle se forme après que la vésicule cristallinienne se soit détachée de l'ectoderme, par la migration des cellules mésenchymateuses périoculaires, issues des crêtes neurales. Le mésenchyme cornéen jouait-il rôle dans la différenciation de l'épithélium cornéen? On sait que tel est le cas pour la majeure partie du tégument, i.e. la peau. Ceci a été démontré maintes fois, depuis les résultats classique obtenus par Dhouailly (1977).

Peau et cornée diffèrent par de nombreux caractères et en particulier par le type de kératines synthétisées. Les kératines, on en denombre plus de 30 principales (Moll et al., 1982), sont les filaments intermédiaires caractéritiques des cellules épithéliales. Elles se répartissent en deux grandes familles, les kératines dites acides, et le kératines dites basiques. Une kératine basique et une acide sont nécessaires à la formation d'hétérodimères qui se groupent ensuite en polymère constituant le filamant de kératine. Les travaux du laboratoire de Sun (1982) ont montré que les couches suprabasales de l'épiderme contiennent la paire de kératines K1, 2/K10, alors que toutes les couches de l'épithélium cornéen contiennent la paire de kératines K3/K12. Sun a avancé l'idée d'une paire de kératines specifiques de la différenciation d'un épithélium donné.

Des travaux récents (Nakamura et al., 2003) montrent qu'en fait K3 est aussi exprimé dans l'épithélium oral. L'expression de la partenaire K12 est-elle réellement restreinte à l'épithélium cornéen? Le gène de la kératine K12 a été montré comme directement régulé par le facteur de transcription Pax6 (Liu et al., 1999). Quel pourait être le rôle de Pax6 lors de la formation de la cornée? Pax6 a été montré par le laboratoire de Goehring à Bâle (1995) comme étant le gène chef d'orchestre (master gene) de la formation de l'œil. Son expression expérimentale dans les disques imaginaux d'antenne, de patte ou d'aile produit des Drosophiles "couvertes " d'yeux ectopiques. Des expériences réalisées durant la progression de mon travail de thèse ont donné des résultats similaires à ceux que j'ai obtenus: chez les

oiseaux, l'expression ectopique de Pax6 n'est pas suffisante pour dèclencher la formation de structures oculaires dans l'etoderme de la tête.

Inversement, des expériences réalisées en collaboration avec David Pearton (Pearton, Yang and Dhouailly, PNAS, 2005, sous presse) ont montré que chez les mammifères, l'expression de Pax6 de l'épithélium cornéen est éteinte lorsque ce dernier est associé à un derme de peau embryonnaire et l'épithélium cornéen est ensuite transformé en épiderme. Le derme embryonnaire a-t-il une action inhibitrice sur l'expression de Pax6, ou bien le stroma cornéen a-t-il une formation activatrice? Les résultats de mes expériences réalisées chez le poulet montreront que la première proposition est en fait exacte. Pax6 étant exprimé à un stade embryonnaire jeune également dans l'épithélium nasal et oral (Tiffany et al., 2002). Nous nous sommes demandé si en associant ces deux types d'épithélium à un stroma cornéen ou pouvait obtenir leur transdifférenciation en épithélium cornéen. Au cours de mes travaux de thèse, le résultat d'expériences semblables aux miennes ont defrayé les médias : Une èquipe japonaise (Nakamura et al., 2003) ayanl affirmé avoir obtenu la transdifférenciation d'épithélium oral en épithélium cornéen. Hors ceci a une grande importance en chirurgie réparatrice humaine. Malheureusement mes propres résultats, obtenus chez le poulet, puis chez les mammifères montrent au contraire l'indépendance des épithéliums nasal, oral et épidermique, qui se différencient selon leur origine et ne sont pas influencés par leur association avec le stroma cornéen.

GENERAL INTRODUCTION

1. The adult cornea.

1. 1 Structure.

One-sixth of the outer layer of the eye (called the tunic fibrosa or fibrous tunic) bulges forward as the *cornea*, the transparent dome which serves as the outer window of the eye. The cornea is the primary (most powerful) structure focusing light entering the eye, along with the secondary focusing structure, the crystalline lens. It is composed, for the most part, of connective tissue with a thin layer of epithelium on the surface. Epithelium is the type of tissue that covers all free body external and internal surfaces.The cornea is composed of 5 layers, from the front to the back: 1- pluristratified epithelium, 2- Bowman's (anterior limiting) membrane, 3- stroma (substantia propria), 4- Descemet's (posterior limiting) membrane, 5- endothelium (**Fig. 1. A-C**). The cornea limbus exists at the interface between the cornea and sclera. It is defined as a narrow ring of tissue situated between the cornea and conjunctiva, terminating centripetally at Bowman's membrane, and centrifugally to 1.5 mm (**Fig. 1. A**).The squamous pluristratified cuboidal epithelium is the cornea's outermost region, about 5-6 layers of cells, in human comprising about 10 percent of the tissue's thickness (**Fig. 1. B and C**). This epithelium functions primarily to: 1- block the passage of foreign material, such as dust, water, and bacteria, into the eye and other layers of the cornea; and 2- provide a smooth surface that absorbs oxygen and cell nutrients from tears, then distributes these nutrients to the rest of the cornea. The corneal epithelium is filled with thousands of tiny nerve endings that make the cornea extremely sensitive to pain when rubbed or scratched. Normally, it is very smooth and lacks of a stratum corneum. The center corneal epithelial basal cells can be identified as transient amplifying cells (TAC) (Schermer et al., 1986; Lehrer et al., 1998; Ferraris et al., 2000; Pearton et al., 2004): they are able to divide, but already

express the corneal specific K12/K3 keratin pair (**Fig. 2**), marker of the terminal step of their differentiation. The distribution of corneal-type keratins was studied by using electrophoretic and immunological analyses with two monoclonal antibodies, specific for K3 (AE5) (Schermer et al., 1986), and for K12 (AK12) (Chaloin-Dufau et al., 1990). Furthermore, Chaloin-Dufau et al. (1993) showed that the acidic keratin K12 is highly conserved throughout all the species studied except the trout, and about the same molecular weight (55-57 kDa). In contrast, the basic keratin K3 shows interspecies variations: it is absent in mouse, while it is present in all other tested species from trout to human, and it or its possible equivalent, vary from 64 kDa in human to 70 kDa in chick. In the chick and in the rabbit, *K12* is expressed in the suprabasal layer of the adult limbus and produces uniform staining of the central corneal epithelium (Chaloin-Dufau et al., 1990). The distribution of K3 is similar and was previously demonstrated in rabbit (Schermer et al., 1986) and in human (Rodrigues et al., 1987). A difference has been shown (Chaloin-Dufau et al., 1990) in the limbal distribution of K3 and K12 in the adult rabbit limbal epithelium. Indeed, the acidic K12 is only synthesized in limbal suprabasal cells close to the cornea, while the basic K3 spreads further throughout the suprabasal cells of the entire limbus.

The extracellular material foundation on which the epithelial cells in general anchor and organize themselves is called the basement membrane and its principal constituants are collagen IV, laminin, and fibronectin. Particularly to the corneal epithelium and lying directly below the corneal epithelial basement membrane is a transparent sheet of extracellular matrix, known as Bowman's layer. It is mostly composed of layered fibers of collagen I and II (Hay, 1980). Once injured, Bowman's layer can form a scar as it heals. If these scars are large and centrally located, some vision loss can occur (Severin and Kirchhof, 1990; Werner et al, 1999; Joyce, 2003). Beneath Bowman's layer is the stroma, which comprises about 90 percent of the cornea's thickness. It consists primarily of collagen I and II, its rare and dispersed

fibroblasts are called keratocytes. The stroma does not contain any blood vessels, it gives the cornea its strength, elasticity, and form. The collagen's fibre unique, regular arrangement and spacing are essential in producing the cornea's light-conducting transparency (Hay, 1980). Under the stroma is the thin Descemet's membrane, a strong sheet that serves as a protective barrier against infection and injuries. Descemet's membrane is composed of collagen fibers II and IV and is synthesized by the endothelial cells that lie below it, and is regenerated readily after injury. The endothelium is the extremely thin, innermost layer of the cornea. Endothelial cells are essential in keeping the cornea clear. Normally, fluid leaks slowly from inside the eye into the middle corneal layer (stroma). The endothelium's primary task is to pump this excess fluid out of the stroma (McCartney, 1987). Without this pumping action, the stroma would swell with water, become hazy, and ultimately opaque. In a healthy eye, a perfect balance is maintained between the fluid moving into the cornea and fluid being pumped out of the cornea. Once endothelium cells are destroyed by disease or trauma, they are lost forever (Elgebaly et al., 1984; Joyce, 2003; Mastropasqua et al., 2004**).** If too many endothelial cells are destroyed, corneal edema and blindness ensue, with corneal transplantation the only available therapy (de Ocampo, 1952).

Thus in brief, the clear and transparent cornea is a highly organized group of cells and extracellular matrix. Unlike the other organs in the body, the cornea contains no blood vessels to nourish or protect it against infection. Instead, the cornea receives its nourishment from the tears and aqueous humor that fills the anterior chamber behind it. The cornea must remain transparent to refract light properly, and the presence of even the tiniest blood vessels can interfere with this process. To see well, all layers of the cornea must be free of any cloudy or opaque areas. On the other hand, the cornea contains the highest concentration of nerve fibers of any body structure. The nerve fibers enter on the margins of the cornea and radiate toward

the center. As in all organs in the body, the cells and extracellular material of the cornea are turned over during the adult life.

1. 2 Corneal Stem cells.

Stem cells can be defined as undifferentiated cells which divide to maintain their numbers, while at the same time producing progeny that proliferate prior to differentiate. Two types of stem cells are distinguished upon their origin / location, as well as the extent of their self-renewal and differentiation potentials: the embryonic and adult stem cells.

Embryonic stem cells (ES) are derived from the inner cells mass of the blastocyst at a stage before it would implant in the uterine wall. In vivo, the ES give rise to all the cells that constitute an embryo. Positional cues trigger cell fate determination that leads to embryonic cell proliferation and morphogenesis as well as differentiation that result in tissue specialisation. In vitro, in given conditions, they can be indefinitely cultured (unlimited self-renewal) or give rise to different cell types which are known to be derived from the three embryonic layers (Bagutti et al., 1996; Coraux et al., 2003; Petitte et al., 2004; Aberdam, 2004). Stem cells generate intermediate progenitor cells whose proliferation and differentiation become more restricted as development cell maturation and specialisation progress, but relatively undifferentiated stem cells appears to be preserved during development, so that stem cells persist into adulthood.

The adult stem cell (AS) will continually replenish lost cells in normal and damaged tissue. Thus adult body has many groups of stem cells existing within it, amongst which are those that reside within the eye. Adult stem cells (AS) share at least two characteristics with the ES. First, they can make identical copies of themselves for long periods of time; this ability is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies (shapes) and specialized functions. Typically, stem cells

which are slow cycling, generate an intermediate cell type or types, the transient amplifying cells (TAC), which actively divide, their descendants achieving their fully differentiated state. The TAC are also called precursor or progenitor cells. Such TACs are usually regarded as "committed" to differentiating along a particular cellular development pathway (**Fig. 3**), although this characteristic may not be as definitive as once thought (Ferraris et al., 2000; Robey, 2000). In contrast to AS, the TAC is more differentiated, has a short life span, is rapid-cycling (Ebato et al., 1988) and can amplify its pool by a limited number of self-replications. TAC has high, but limited proliferative activity (Costarelis et al., 1989; Pellegrini et al., 1999). Adult stem cells (AS) are rare. Their primary functions are to maintain the steady state functioning of a cell-called homeostasis and to replace cells that die because of injury or disease (Holtzer, 1978; Leblond, 1966). For example, in human only an estimated 1 in 10.000 to 15.000 cells in the bone marrow is a hematopoietic stem cell (Vescovi et al., 1993). Furthermore, AS are dispersed in tissues throughout the mature animal and behave very differently, depending on their local environment (Domen and Weissman, 1999). In brief, the AS is an undifferentiated (unspecialized) cell that is found in a differentiated (specialized) tissue; it can renew itself for the lifetime of the organism. For example, the corneal epithelium renewed in 3-4 weeks in adult mouse (Collinson et al., 2002). In a tissue, the AS is at the source of all of the specialized cell types of the tissue from which it localized. For example, the stem cells localized in hair follicles are able to give rise not only to the seven different cell types of the hair follicle itself, but also to the sweat gland, sebaceous gland and epidermal cells (Oshima et al., 2001). Sources of AS have been found in bone marrow, corneal epithelium and retina of the eye, dental pulp of the tooth, liver, epidermis, gastrointestinal epithelium, pancreas and brain. Unlike ES, at this point in time, there are no isolated AS that are capable in experiments of forming all cells of the body. That is, there is no evidence, at this time, of an AS that is pluripotent (Chandross and Mezey, 2001; Slack,

2000). Futhermore, a question arises: are differently localized AS equivalent, similar or quite different?

Adult stem cell fate, whether it is quiescence, apoptosis, division or differentiation is believed to be under the control of interactions between the AS and its microenvironment or niche (among others: Morrison et al., 1997; Watt and Hogan, 2000). The AS is likely to spend part of it's life withdrawn from the cell cycle in the resting state (Go) (Cotsarelis et al., 1989; Lajtha, 1997), thereby reducing the risk of mutagenic insults. When the AS exits its compartment, the factors that maintain its 'stemness' are no longer available, and the cell is likely to enter the differentiation pathway as a result of new local environmental influences. Various factors, short and long range, internal and external are believed to be involved in the control of AS survival, proliferation and differentiation across a number of tissues. External controllers include: secreted factors, cell-cell interactions, extracellular matrix and cell-adhesion molecules (among others: Conlon and Raff, 1999; Morrison et al., 1997; Watt and Hogan, 2000).

There is no current definitive biological cell marker for stem cells. By studying the cornea model, Pellegrini et al. (2001) proposed the transcription factor P63 as a potential keratinocyte stem cell marker. However, p63 did not appear to be exclusive to stem cells, as although absent in cultured paraclones (TAC), p63 was identified at low levels (compared to those holoclones/ stem cells), in meroclones (young TAC) also.

The primary source of corneal epithelium, a population of stem cells, are believed to reside in the limbal region (**Fig. 2**), and give rise to transient amplifying cells (TAC) which are in the center of the cornea (Dua and Azura-Blanco, 2000a,b; Kruse, 1997). Almost 10% of limbal basal cells might be AS (Lavker et al., 1991). Limbal epithelial stem cells phenotypically appear more primitive than corneal epithelial cells, being small and round (Lavker et al., 1991). Several studies have documented their lack of differentiation: the

keratin 3 (K3) was observed in all layers of the corneal epithelium, but only in the suprabasal layers of the limbus (Schermer et al., 1986). A similar pattern of localisation was demonstrated for keratin 12 (K12), adding further evidence of a population of least differentiated cells in the corneal limbus (Chaloin-Dufau et al., 1990; Kurpakus et al., 1990). Thus, the underlying limbal stroma and cells within, as well as the local blood supply, are all likely to contain factors that determine AS fate. The limbal AS are undifferentiated, slow-cycling cells that self-renew and produce TAC which are considered to migrate centripetally to the corneal epithelium (Kinoshita et al., 1981; Tseng, 1989). They can be localised to the Palisades of Vogt in the limbus (Davenger and Evenson, 1971). These authors showed the migration of pigmented cells from the limbus towards the central cornea in a wound healing response. Several studies have demonstrated that limbal epithelial cells have a higher proliferative potential than those from peripheral and central cornea (Lavker et al., 1991; Ebato et al., 1988). Clonogenicity studies have confirmed that holoclones (derived from stem cells) have the greatest capacity for clonal expansion (Pellegrini et al., 1999). Interestingly, the limbal stem cell presence appears to vary in different quadrants of the eye, being greater in the superior and inferior cornea in comparison to nasal and temporal meridians (Lauweryns et al., 1993; Wiley et al., 1991). As the TAC moves across the limbal-corneal margin, the cell develops differentiation features: keratins 3 and 12 (Kurpakus et al., 1990; Chaloin-Dufau et al., 1990; Schermer et al., 1986). A peak proliferative activity occurred in limbal zone and the mitotic index also appears to be under the influence of the circadium cycle (Lavker et al., 1991).

The precise number of times that a TAC divides is dependent upon requirement: both cell cycle number and frequency are upregulated in corneal wound healing (Cotsarelis et al., 1989; Lehrer et al., 1998; Lindberg et al, 1993). Not only do stem cells ensure that the corneal epithelium undergoes continual self-renewal, they are also responsible for epithelial tissue

repair and regeneration throughout the life (Daniels et al., 2001; Dua and Azuaro-Blanco, 2000a,b; Miller et al., 1993; Kinoshita et al., 2001; Tseng and Sun, 1989). Under normal physiological conditions limbal epithelial stem cells and central TAC are able to meet the demand required to maintain normal epithelial homeostasis. In some pathological states, an absence or depleted supply of limbal epithelium fails to maintain a stratified corneal epithelium. This results in conjunctivalisation of the ocular surface, persistent epithelial defects, neovascularisation, scarring, ulceration and eventual corneal perforation. Such pathologies include traumatic injuries such as chemical and thermal burns, contact lens-induced keratopathy, Stevens Johnson syndrome and ocular pemphigoid (Chiou et al., 1998; Wagoner, 1997). It can be considered that limbal AS constitute a reservoir which is activated when wound healing of corneal epithelium is required.

Additional data support for the limbal location of corneal epithelium AS. Limbal transplants have the ability to generate a stratified corneal epithelium (Dua and Azuaro-Blanco, 2000a,b). Moreover, a corneal epithelium can not form in the absence of limbal epithelium (Chen and Tseng, 1990, 1991; Huang and Tseng, 1990, 1991; Kruse et al., 1990), although this appears to be contradicted by recent findings of Dr. Barrandon laboratory (personal communication).

A recent (Collison et al., 2004) publication of Dr. West group provides a strong argument in favour of the centripetal migration of limbal epithelium cells. This group had previously shown that the production of chimeras by aggregation of two eight-cell mouse embryos, in which one of the contributing embryos carries a marker (LacZ) by which its cells can be identified, is an established method for clonal analysis of growth patterns in the mouse (West, 1999). Likewise, female mice that carry a marker transgene on the X chromosome, which is subject to random X-inactivation, may produce mosaic patterns of transgene activity that also allow investigation of cell lineages (Tan and Breen, 1993). Thus, Collinson et al.

(2002) investigated patterns of growth and cell movement in the developing and adult corneal epithelium by analysing clonal patches of $LacZ^-$ expressing cells in chimeric and X-inactivation mosaic mice (**Fig. 4**). It was found that cell proliferation occurs throughout the entire basal layer of corneal epithelium during embryogenesis, and this gives rise to a mosaic pattern. At postnatal stages (pns), an important change occurs. A series of 21 $LacZ^+ \Leftrightarrow LacZ^-$ chimeric mice was analysed at 18-32 weeks pns. X-Gal staining revealed a variety of radial patterns of blue and white stripes (**Fig. 4. A-F**). Infrequently, bilateral symmetry was shown, where stripes appeared to meet at a midline rather than at the centre. Histology revealed that the blue staining was limited to the corneal epithelium (not penetrating Bowman's membrane into the stroma) (**Fig. 4. H**). Basal cells were normally the same colour as the more apical cells above them, suggesting that they are clonally related, although occasional exceptions were noted. In addition, their data show that there is little mixing between the radial streams of clonally related cells. There was often a visible swirl of cells at the centre of the cornea, where the migrating streams meet (**Fig. 4. G**). Consequently Collison et al. propose a model of corneal maintenance (**Fig. 5**) that confirms, as previously thought, a radial migration of transient amplifying progeny of limbal stem cells in the basal layer of the corneal epithelium, with differentiation occurring as cells lose contact with the basal membrane and move apically.

Limbal stem cells were believed until recently to be unipotent. The fact that adult basal corneal epithelial cells (TAC) have been shown to alter phenotype i.e. to transdifferentiate into epidermal cells, in response to embryonic dermis (Ferraris et al., 2000; Pearton et al., 2004), suggest that epithelial cells can be reprogrammed in accordance with the 'screw' model of stem cell behaviour of C. Potten. This model suggests that TAC, that have started the differentiation process, can be induced under certain signals to revert to their original 'stemness' (Loeffler et al., 1997; Potten and Loeffler, 1990). Following maturation of these

de-differentiated stem-like cells, they appear able to participate in hair-follicle in response to their new niche, and finally to express epidermal specific keratin 10 (Ferraris et al., 2000; Pearton et al., 2004). This new model of AS behaviour proposed by D. Pearton is presented on **Figure 6**.

2. Development and structure of other head epithelia.

2. 1 Epidermis.

2. 1-1 Introduction.

In vertebrates, the ectoderm covers the entire embryo after neurulation. Originally, one cell-layer thick, the ectoderm shortly becomes a two-layered structure: it divides, early on, into a cuboidal germinal layer, the epidermis and an outer flat layer, the periderm. The periderm protects the embryo during its growth as its cells die, keratinize, and it is sloughed a few days before birth. The cuboidal layer beneath forms the basal layer of the epidermis that gives rise to all the cells of the epidermis which is several cells thick by the time of birth. The cells of the basal layer are bound to the basement membrane by their integrin proteins. However, as they become committed to differentiate, they downregulate their integrins and lose them as the cells migrate into the spinous layer (Jones and Watt, 1993). These two epidermal strata, the basal layer and spinous layer, are referred to as the Malpighian layer. As these cells mature and are carried towards the surface of the skin (**Fig. 7. A -D**) forming the stratum corneum, in which the cells have become flattened sacs of keratins and various associated proteins. In chick feathered areas the Malpighian layer is only two or three cells thick (**Fig. 7. A**), whereas in other areas this layer is much thicker, furthermore on the feet the epidermis is folded into scales. A similar regional variation occurs in mammals that show in particular a thick plantar epidermis. The K1/K10 epidermal–specific keratin pair is expressed

in suprabasal differentiating cells (Sun et al., 1983) (**Fig. 7. B** and **D**). The K5/ K14 pair of keratins is expressed in all the basal layer cells of the epidermis, this pair being characteristic of basal cells in all the stratified epithelia (Sun et al., 1983), except the center cornea. In mammals, in contrast with the birds, the cells of Malpighian layer before to form the stratum corneum differentiate into the granular layer (**Fig. 7. C**), so called because the cells are characterized by granules of profilaggrin. The depth of the cornified layer varies from site to site, but it is usually 10-30 cells thick in mammals.

Throughout life, the dead keratinized cells of the cornified layer are being shed (we humans lose about 1.5 grams each day) and are replaced by new cells, the source of which is the mitotic cells of the basal layer. In wounding consisting in superficial skin abrasion, sparing the upper part of hair follicles, the epidermis is regenerated by islands of cells emerging from the hair follicle bulge (stem cell reservoir).

2. 1-2 Role of the dermis in adult epidermal differentiation.

Saiag et al. (1985) used a skin equivalent model to fabricate tissues with human psoriatic and normal cells. They showed that psoriatic fibroblasts can induce hyperproliferative activity in normal keratinocytes. The psoriatic epidermis from lesions continues to proliferate at high rates for at least 15 days in this model, and normal fibroblasts are unable to suppress this hyperproliferation. So they concluded that the primary defect in psoriatic skin may reside in the dermal fibroblast. Another example in clinic is to make artificial skin for graft to patients. From 1981 Coulomb et al. (1998) grafted 18 patients (burns and giant nevi) using 35 grafts 10x10 cm in size. In the course of this work, the original technique was modified and improved as experience was gained. They began by using small skin biopsy samples as a source of keratinocytes cultured on a dermal equivalent before grafting in a one-step procedure, but this gave poor cosmetic results, because of a

nonhomogeneous epidermalisation. In a second time they chose to cover the graft bed using a two-step procedure. The first step consisted of grafting a dermal equivalent to provide a dermal fibroblast-seeded substrate for subsequent in vivo epidermalisation by cultured epidermal sheets. Whatever the epidermalisation technique used, a living dermal equivalent applied to the graft bed was found to reduce pain, to provide good hemostasis, and to improve the mechanical and cosmetic properties of the graft. A normal undulating dermal-epidermal junction reappeared by 3 to 4 months after grafting and elastic fibers were detectable 6 to 9 months after grafting. Another example which culture is used to transfer to clinical use is showed in Dr. Barrandon's laboratory (Ronfard et al., 2000). The human keratinocytes cultured on a fibrin matrix had the same growth capacity and transplantability as those cultured on plastic surfaces and the presence of a fibrin matrix greatly facilitate the preparation, handling, and surgical transplantation of the grafts, which did not need to be detached enzymatically. The rate of taking off grafts grown on fibrin matrices was high, and was similar to that of conventionally cultured grafts. The grafted autologous cells are capable of generating a normal epidermis for many years showing thus a restoration of the stem cell population and favor the regeneration of a superficial dermis. Even though artificial skin is achieved for grafting to the patients, nobody can make perfect artificial skin exactly like normal tissue with sweat glands, hair follicles, and skin elasticity. Recently, Navsaria et al. (2004) reported a head and neck full-thickness burn injury that was reconstructed with a tissue-engineered dermal template and then early implantation of microdissected hair follicles through the silicone epidermis 12 days after the burn injury. The treatment resulted in complete reepithelialization and a hair-bearing scalp without the need for a split-thickness skin graft. Hair growth, and earlier reepithelialization were achieved using this novel micrografting technique, and histologic examination confirmed maturation of a normal skin type over the subsequent 2 years.

2. 1-3 Role of the dermis in embryonic epidermal differentiation.

The dermis has different embryological origins: the mesoderm for the body, the neural crest for the face and most part of the scalp. The epidermis and dermis interact during embryogenesis at specific sites to create the sweat glands and the cutaneous appendages: hair, scales, or feathers, depending on the species (Dhouailly, 1977). The first indication that a hair follicle will form at a particular place is a thickening of cells in the basal layer of the epidermis, forming a placode. This formation is directed by the underlying dermal cells and occurs at different times and different places in the embryo and the dermal cells respond to this ingression of basal epidermal cells by forming a small aggregation, the dermal condensation, beneath the placode (Dhouailly, 1977). In mammals, the basal cells of the placode elongate, divide, and sink into the dermis to form the hair peg. There are many signals which mediate dermal-epidermal interactions and which have been mostly studied in the chick model (see as a review: Olivera-Martinez et al., 2004) (**Fig. 8**). Among these signals, the Wnt pathway is associated first in the formation of the dorsal dermis. The dermal precursors express Wnt11, which might be implicated in their migration to the subectodermal space (Olivera-Martinez et al., 2001, 2002). At early stage the level of β-catenin is homogeneous throughtout feather field epidermis and then is restricted to placodes (Widelitz et al., 2000). This message appears to become restricted to the primordia in wild-type embryo, whereas it remains as a smear over the tract fields in the scaleless embryo which is deprived of placodes and do not form feathers or scales (Widelitz et al., 2000). During pattern formation nuclear β-catenin staining increases in the placode and is lost in the ectoderm that adopts interfollicular fate. In addition the forced expression of β-catenin induces the formation of ectopic feathers (Noramly et al., 1999; Widelitz et al., 2000). β-catenin, an effector of intercellular adhesion, also functions in Wnt signaling, associating with Lef-1/Tcf

DNA-binding proteins to form a transcription factor. Gat et al. (1998) reported that this pathway operated in keratinocytes and that mice expressing a stabilized β-catenin controlled by an epidermal *K5* promoter undergo de novo hair morphogenesis. The new follicles formed sebaceous glands and dermal papilla, normally established only in embryogenesis. Additionally, proliferation continues unchecked, resulting in tumors, which are known in humans, and called pilomatricoma. Mutations of the *β-catenin* gene are detected in 75% of the tumours analysed (Durand and Moles, 1999). Their findings suggest that transient β-catenin stabilization may be a key player in the long-sought epidermal signal leading to hair development and implicate aberrant *β-catenin* activation in hair tumors. The restriction of *β-catenin* expression, as well as that of *Delta-1* expression might be a consequence of *FGF4* expression in the epidermis (Viallet et al., 1998; Song et al., 1996). The Notch pathway may serve to stabilize the patterning of feather primordia (Viallet et al., 1998). The coupled BMP4/BMP-antagonist is first observed during feather field specification in the abdomen (Fliniaux et al., 2004). During pteryla formation, transient *BMP2* expression is observed in the epidermis while the BMP antagonists gremlin and follistatin are expressed in the underlying dermis and the epidermis, respectively (Noramly et al., 1998). When the patterning occurs, BMP2, 4 and 7 and the BMP antagonists excepted gremlin are expressed in the primordia. These observations lead to a model based on activation via differential diffusion of activators and inhibitors for the formation of the periodic patterning (Jiang et al., 1999). A primordia comprises a placode: a thickening of the epidermis, overlying a dermal condensation.

In mammals, the dermal condensation that gives rise to the dermal papilla then pushes up and stimulates the basal epidermal cells to divide rapidly and to produce postmitotic cells that will differentiate into the keratinized hair shaft (Dhouailly, 1977; Miller et al., 1993).

2. 1-4 Role of the dermal papilla in inducing hair in an adult epidermis.

Dr. Jahoda (1992) described how the implantation of isolated vibrissa papillae into small incisional cuts on the rat ear pinna result in the subsequent emergence of abnormally large hair fibres from the wound sites. Many of these hairs were found to display vibrissa-type characteristics. Histological observations indicated that the papillae had interacted with the edges of the wound epidermis to produce new and particularly large follicles, while immunohistochemical staining revealed that early follicle construction was accompanied by a profusion of the basement membrane constituent laminin and type IV collagen in the subjacent dermis. These findings show that adult rat papillae retain the capacity, as displayed by embryonic dermis (Dhouailly, 1977), to determine vibrissa specificity in induced follicles. To test the inductive and immunoreactive properties of human hair-follicle dermis, Reynold et al. (1999) microdissected dermal sheath tissue from the base of scalp skin follicles of one of them (C.A.B.J), a male. They implanted this tissue into shallow skin wounds on the inner forearm of another one of them (A.J.R), a genetically unrelated and immunologically incompatible female recipient. Five months later, the same female received grafts of follicle dermal sheath and dermal papillae from the same male donor and, in a third experiment, dermal sheath from a second unrelated male donor. All the wound sites healed rapidly and lacked any overt inflammatory reaction. Remarkably, each of the sites of dermal-sheath implantation produced new follicles and fibres 3 to 5 weeks after graft. Unlike the tiny, unpigmented vellus hair of the host arm, the newly induced hairs were larger and thicker, mostly pigmented, and grew in variable directions. None of the new follicles show the evidence of rejection when biopsied between 41 and 77 days after the graft. Histology confirmed that the new follicles were morphologically normal, with oval dermal papillae overlaid by a pigmented epidermal matrix at their base. Several experiments of DNA analysing, confirmed that the DNA extracted from the papillae cells had an X and Y

complement, whereas cells taken from other regions of the same follicle and epidermis in the immediate vicinity had only X chromosomes and so were female. Their results show that follicle dermal cells from a human adult can initiate epithelia-mesenchymal interactions and create new follicles without being rejected. Melanoblasts, which were present among the epidermal cells as they ingressed, differentiate into melanocytes and transfer their pigment to the shaft.

2. 1-5 Hair follicle and epidermal adult stem cells.

The hair follicle present two swellings: the cells of the lower swelling, the bulb, may retain a population of stem cells that will allow the growth of the hair shaft (Cotsarelis et al., 1990). The cells of the upper swelling of the outer root sheath constitute the bulge (not morphologically individualized in human). To further investigate the fate of the cells located in this region, Oshima et al. (2001) of Dr. Barrandon laboratory implanted individual fragments of bulges obtained from vibrissal follicles of adult Rosa 26 mice onto the back of OF1 mouse embryos. They report that the upper region of the vibrissal follicle of the adult mouse are the reservoir of adult epidermal stem cells (AS) that periodically respond to morphogenetic signals by generating hair follicles, sebaceous glands, and epidermis. To further evaluate the role of these AS and that of the clonogenic keratinocytes in hair renewal, they performed clonal analyses and transplantation of different parts of vibrissal follicles obtained at different phases of the hair cycle. They demonstrate that the distribution of the clonogenic keratinocytes is greatly influenced by the hair cycle, and that the lower part of a vibrissal follicle can respond to morphogenetic signals only when it contains a significant number of clonogenic keratinocytes. Their results showed that multipotent stem cells migrate to participate in hair renewal and strongly suggest that the clonogenic keratinocytes and multipotent stem cells are closely related, if not identical. Moreover, Oshima et al. (2001), as

well as Ferraris et al. (2000), Pearton et al. (2004) showed that the hair stem cells are the reservoir for the epidermal stem cells. However, it should be noted that epidermal AS are also dispersed throughout all the basal layer of the epidermis (**Fig. 7. D**).

2. 1-6 Main differences between corneal epithelium and epidermis.

In brief, facial skin and cornea are epithelial–mesenchymal organs composed of an ectoderm-derived stratified epithelium and of a neural-crest derived mesenchyme (Couly and Douarin, 1988; Osumi-Yamashita et al., 1994). The epidermis (**Fig. 7**) and the corneal epithelium (**Fig. 2**) can be distinguished by four major characteristics: 1- the presence or absence of a stratum corneum 2- the formation or not of appendages such as hair follicles or feathers 3- the expression of different pairs of keratins 4- and the localization of their stem cells. In the epidermis, as in most stratified epithelia, the stem cells occupy specific location within the entire basal layer (Lavker and Sun, 1982; Jensen et al., 1999).

Keratins are the intermediate filaments that are characteristic of epithelial cells. Keratins represent a family of more than 30 proteins (Moll et al., 1982). Different subsets of keratins are expressed in different epithelia and there exist a number of rules governing keratin expression. They first involve two subfamilies, clarified by their charge into an "acidic" and a "basic" subfamily. The formation of a filament requires the interaction of an acidic and a basic keratins, that constitutes a "keratin pair". In stratified epithelia, the basal cells are relatively undifferentiated and express the K5/K14 pair, whereas the suprabasal differentiating cells express a keratin pair that varies and is considered (Sun et al., 1983) as a marker for skin (K1,2/K10), cornea (K3/K12) or esophagus (K4/K13). The K1/ K10 epidermal –specific keratin pair is expressed in suprabasal differentiating cells (Sun et al. 1983), as well as in 8 to 10% of the basal subpopulation which has already embarked on terminal differentiation (Régnier et al., 1986; Schweizer et al., 1984). In epidermis, most of the basal layer expresses

the K5/K14 keratin pair. In contrast, all corneal epithelial basal cells can be identified as transient amplifying cells: they are able to divide but already express the corneal specific K3/K12 keratin pair, marker of the terminal step of their differentiation (Schermer et al., 1986; Chaloin Dufau et al., 1990). The corneal stem cells that do not express this keratin pair but the K5/K14 appear to be located at the periphery of the cornea in the basal cells of the limbal epithelium (Chaloin-Dufau et al., 1990; Cotsarelis et al., 1989; Lavker et al., 1991; Pearton, Yang et al., 2004).

2. 2 Conjunctival epithelium.

The formation of the conjunctiva during development was never studied. In mammals, the conjunctival epithelium is a thin stratified squamous epithelium consisting of two cell types: epithelial cells with goblet cells interspersed, which overlaid a vascularised stroma. These two cell types are believed to originate from a common precursor cell (Wei et al., 1997). The goblet cells are crucial for ocular surface integrity. The conjunctival epithelial (**Fig. 9. D**) is divided in three regions: the bulbar, fornix and palpebral conjunctiva. Keratin-4 and keratin-13, which were primiraly considered as esophagal-type keratins (Sun et al., 1983), are expressed in the superficial and intermediate layers of the conjunctiva (Nakamura et al., 2003).

Like other epithelia, the conjunctival epithelial cells are constantly renewed. Wirstschafter et al. (1999) demonstrated the presence of slow-cycling label-retaining cells in the rabbit palpebral conjunctiva, close to the mucocutaneous junction. More recently, slow-cycling cells were localised in palpebral conjunctiva, these epithelial cells showing a greater proliferative capacity than epithelial cells from other conjunctival regions (Chen et al., 2003). This suggests palpebral conjunctiva as a stem cell reservoir for the conjunctival epithelium,

and perhaps for the corneal cells as suggested by recent results of Dr. Barrandon laboratory (personal communication).

2. 3 Oral epithelia.

The developmental origin of the epithelium lining the oral cavity is generally thought to be ectoderm anteriorly and endoderm posteriorly. The oral cavity is formed by the lips, cheeks, hard and soft palate, and the floor of the mouth. In mammals, the oral cavity is divided in a vestibule, the area "outside" the teeth, and an oral cavity proper. The entire oral cavity is lined by a stratified squamous epithelium (**Fig. 9. A and C**). This epithelium is divided into two broad types: masticatory epithelium and lining epithelium. Masticatory epithelium covers the surfaces involved in the processing of food (tongue, gingivae and hard palate). The epithelium is keratinized to different degrees depending on the extent of physical forces exerted on it. Keratinized stratified squamous epithelium covers stressed areas, including hard palate, gingivae, and parts of the dorsal surface of the tongue. Stratified squamous epithelium is very proliferative. It has to be; its cells are "sacrificial" and sloughed constantly as they die and are abraded away. They are renewed by constant mitosis in the bottom layer. Lining epithelium, is non-keratinised stratified squamous epithelium, covers the remaining surfaces of the oral cavity, including inside of the lips, cheeks, soft palate, floor of the mouth, lower surface of the tongue and tonsils.

For keratin expression, not only keratin-4 and keratin-13 characteristic of the esophagal epithelium (Sun et al., 1983) are expressed in all epithelial layers of oral epithelium, but also the corneal type keratin-3, but not K12, as it is was recently shown (Nakamura et al., 2003). Moreover, the mouse and human tongue filiform papillae express hair keratins (Dhouailly et al., 1989).

2. 4 Nasal epithelia.

In mammals, the cavernous nasal cavity is separated by the median nasal septum for superior, middle, and inferior conchae. Extending from both lateral walls are three curved plates of bone covered by mucous membrane. Respiratory epithelium covers inferior and middle conchae and is of ectodermal origin. Olfactory epithelium, which embryonic origin has not yet be carefully studied, might be in part of endodermal origin like the lungs, and extends laterally overly the superior conchae and medially over the superior portion of the nasal septum. It is composed of a tall, pseudostratified epithelium (**Fig. 9. A** and **B**) with goblet cells. Olfactory epithelium includes three cell types: olfactory (sensory) cells, supporting (sustentacular) cells, and basal cells. Olfactory cells are bipolar neurons, which dendrite reaching the surface forms an apical knob. The basal portion of the cells extend as unmyelinated axons which aggregate as small nerve directed toward the central neural system and traverse the cribriform plate. Cilia of sensory cells increase surface area and are sensitive to chemical stimuli. Secretions cleanse epithelial surface keeping receptors ready for new stimuli. Supporting cells are columnar and contain a yellow pigment. They are covered with luminal microvilli. Basal cells are small round cells and are thought to give rise to new sustentacular and olfactory sensory cells, and might correspond to stem and TAC cells.

3. Eye development and corneal epithelium differentiation.

3. 1 Dynamic of eye development.

Optic development begins at gastrulation when the involuting endoderm and mesoderm

interact with the adjacent prospective head ectoderm. The interaction gives a lens-forming bias to the head ectoderm (Saha et al., 1989). The eye development will then involves cell interactions between two epithelia, the ectoderm and the neuroderm of the optic vesicle, and one mesenchyme that originates from the neural crest. The activation of the latent lens-forming ability of the ectoderm and the position of the lens in the relation to the retina is accomplished by the neuroderm of the optic vesicle. Indeed, when this vesicle contacts the head ectoderm, the ectoderm thickens into the lens placode. Spemann (1901) obtained an ectopic lens by working in amphibian and grafting the optic vesicle beneath the ventral abdominal ectoderm in amphibian embryo. It was the first time that the differentiation of a tissue, i. e. the ectoderm, was shown to be controled by an other tissue, i.e. the neuroderm of the optic vesicle. The result of Spemann's experiment, that triggers all the field of experimental embryology, however was wrong. Indeed, at the beginning of the nineties, this experiment was repeated, using as a donor an embryo labeled with fluorescein-dextran (FLdx). Only a few cases of lens induction were obtained, and the lens was also fluorescent, showing that some ectodermal cells of the donor were dissected together with the optic vesicle. In fact, the ability to form a lens in response to the optic vesicle induction appears restricted to part of the head ectoderm (for a review: Grainger, 1992). The necessity for close contact between the optic vesicle and the surface ectoderm is seen in both experimental cases and in certain mutants. For example, in mouse mutant small eye, the optic vesicle fail to contact the surface and eye formation ceases (Webster et al., 1984).

Once formed, the lens placode reciprocates and causes changes in the optic vesicle. The vesicle invaginates to form a double-walled optic cup. As the invagination continues, the connection between the optic cup and the brain is reduced to a narrow slit. At the same time, the two layers of the optic cup begin to differentiate in different directions. The cells of the outer layer produce pigment and ultimately become the pigmented retina. The cells of the

inner layer proliferate rapidly and generate a variety of light-sensitive photoreceptor neurons, glia, interneurons, and ganglion cells. Collectively, these cells constitute the neural retina. The axons from the ganglion cells of the neural retina meet at the base of the eye and travel down the optic stalk. The stalk is then called the optic nerve.

During its continuted development into a lens, the lens placode invaginates, rounds up and contacts the new overlying ectoderm (**Fig. 10. A-C**). The lens vesicle then is believed to induce the ectoderm to form the transparent cornea (Hay, 1980). Directly in front of the lens is a pigmented tissue: the iris. Its muscles control the size of the pupil. Unlike the other muscles of the body which are derived from the mesoderm, the iris is derived from the neuroderm layer (Gilbert et al., 1991).

3. 2 Cornea differentiation.

The cornea of all vertebrates begins its development in the ectoderm overlying the lens. The following description is done by comparing the observations in **rabbit** by one previous student in this laboratory (Chaloin-Dufau et al., 1990) and for the **chick**, my observations as well as the previous work of Hay (1980). The anterior corneal epithelium begins to form from the ectoderm which reunites after the lens vesicle has separated from it, at 2.5 days of incubation in the chick (**Fig. 10. A-C**) and at 11.5 days of gestation in the rabbit. Then at 4.5/5 days of incubation, the development of the avian cornea is charaterized by the formation of an acellular stroma on the fourth/fifth day (**Fig. 10. D-F**), whereas in rabbit, at the corresponding developmental stage (13 days), the stroma is already produced by residential fibroblasts. At this stage, in both species, the corneal epithelium consists of two cellular layers, the periderm and the ectoderm proper. Then in chick, a second wave of fibroblasts migration invades all layers of the stroma at 6.5/7 days of incubation (**Fig. 10. G-I**). By 10 days in chick embryo and 17 days in rabbit embryo, the cornea is well defined and the eye is

characterized by the formation of both the anterior chamber and the lid buds. By 13 days (chick embryo) and 21 days (rabbit embryo), the corneal epithelium starts to stratify, during which time the lids close. At this stage, most of the differentiation of the avian corneal stroma takes place: these include the formation of the Bowman's and the Descemet's membranes and the condensation (dehydration) step which begins in the posterior stroma. A similar differentiation of an anterior and posterior stroma occurs in the rabbit at 21 days. By 15 days, the corneal epithelium has become four or five cells layers thick in chick embryo, while in 23-days rabbit embryo it is only three or four cell layers thick. At this stage, the compaction of the posterior stroma is obvious. At birth, the chicken has its eye opened and its corneal epithelium contains four or five living cells layers, covered by two to three flat cell layers (**Fig. 1. C**). In contrast, the newborn rabbit is still immature: the maturation of the cornea, i.e. the increase in thickness of its corneal epithelium will occur around the period of the eye opening, 12 days post-natally (Chaloin-Dufau et al., 1990).

In **mouse**, the development of the anterior segment of the eye begins at about embryonic day 11 when the lens vesicle closes and separates from the surface ectoderm (Pei and Rhodin, 1970). The surface ectoderm restores its continuity and forms the corneal epithelium. Beginning at 12 days of gestation, mesenchymal cells of neural crest origin invade the space underneath the corneal epithelium and anterior to the lens vesicle. It was recently shown in Dr. P. Chambon laboratory (Dr. N. Matt, personal communication) that the corneal epithelium expresses the raldh enzymes which transform retinaldehyde into retinoic acid that allows the migration of neural crest cells mesenchyme. By 15 days, these mesenchymal cells differentiate into corneal stromal fibroblasts and a sheet of corneal endothelial cells lining the posterior (inner) border of the cornea. During this time, a definitive anterior chamber forms, which creates a space between the lens and cornea. By 17 days, the ciliary body begins to differentiate from neurectoderm at the base of the iris, and the iris begins to elongate and

extend into the space between the lens and cornea (Reneker et al., 2000). At birth (19 days), the eyes are still closed.

So, in chick the corneal ectoderm first produces a highly organized stroma that is subsequently invaded by fibroblasts, whereas in mouse and rabbit, the first highly ordered corneal stroma appears to be produced by the fibroblasts (Hay, 1980).

In **human** embryo, at an early development stage (30 days, 6mm long: crown to rump length) the eye development is similar to that observed in 2.5 days chick embryo. The formation of an acellular corneal stroma, bounded by epithelium and endothelium, prior to fibroblast invasion has been reported in both human (Düblin, 1970; Ewer, 1970; Mizutani, 1976) and monkey embryos (Ozanics et al., 1977). This primary stroma, is not as well organized as in birds, being composed of fine filaments and amorphous materials with only a few orthogonal collagen fibrils (Mizutani, 1976; Ozanics et al., 1977). In the human and other primates, two successive waves of mesenchymal cells are said to form the corneal endothelium and then the keratocytes (Düblin, 1970; Ewer, 1970; Ozanics et al., 1977). The secondary mesenchymal stream enters the human cornea at the beginning of the second month (23-25mm). Corneal swelling preceding the invasion of fibroblasts has been described in the monkey (Ozanics et al., 1977) and probably also occurs in human. In the period around the third month (30-40mm), the invasion of fibroblasts into the human cornea is completed. The posterior mesenchyme or mesenchymal shelf is closely related to the optic cup vasculature and later forms the pupillary membrane (Ewer, 1970; Düblin, 1970). The endothelium is continuous with the trabecular meshwork which appears subsequently (Smelser and Ozanics, 1971; Zinn and Mockel-Pohl, 1975).

After the fibroblasts enlarge the developing human cornea (45mm), the posterior stroma condenses (Murakami et al., 1970; Leone-Messeni, 1973). The posterior fibroblasts become flattened, much as in the avian eye at 14 days, but the relation of the stromal compaction to

the beginning of corneal dehydration and transparency, shown in chick (Coulombre and Coulombre, 1958b, 1964) has not been worked out for the primate eye (Ozanics et al., 1976, 1977). As in the chick, the collagen fibrils of the developing human cornea are narrow (30nm in diameter; Schwarz, 1953, 1961). Descemet's and Bowman's membranes form between the third and eighth month (Ozanics et al., 1976, 1977). Corneal diameter reaches adult size a few years after birth (Ehlers et al., 1968).

3. 3 Corneal epithelium differentiation and functions.

In 2.5 days chick embryo, the corneal epithelium is two cells thick, involving a flattened layer, the periderm, overlying a basal cuboidal layer of cells, the proper corneal epithelium. In chick, these basal cells become columnar in shape (Hay and Revel, 1969) between stage 18 and 22 of Hamburger and Hamilton (1951) (around 3 days), and the corneal epithelium begins to stratify at stages 35-36 (9-10 days). By 14 days of incubation, it is four cells thick.

The secretory activities of the isolated corneal epithelium to deposit the primary stroma are believed to occur between 4 and 5 days in chick embryo (Trelstad, 1970) and have not been studied in detail in other vertebrate species. Rabbit corneal epithelium has been noted to produce stromal extracellular matrix (ECM) in vivo during regeneration (Leuenberger et al., 1973; Leuenberger and Gnädinger, 1972). As in the case of chick, rabbit corneal epithelium secretes glycosaminoglycans, but not hyaluronate, whereas the endothelium produces hyaluronic acid (Gnädinger and Schwager-Hugner, 1975). Isolated human epidermis can produce basal lamina in vitro when grown on frozen-killed dermis (Briggaman et al., 1971). It may be a general rule that the initial ECM of various vertebrates cornea is mainly epithelial in origin (Hay, 1980).

At the beginning of the period of epithelial stratification, by day 10 of incubation in chick, the first nerves traverse the stroma to enter the corneal epithelium. In monkey, corneal

intraepithelial nerves appear at the end of the second month while the epithelium is still two layered, this later stratifies subsequently (Ozanics et al., 1977). The intraepithelial nerves are unmyelinated and tend to be enclosed by epithelial gap junctions (Segawa, 1964; Hay and Revel, 1969; Ozanics et al., 1977). Because in the embryo epithelial stratification in the cornea precedes that of the epidermis, it would be interesting to explore the possibility that the nerves trigger the precocious corneal stratification.

The role of epidermal growth factor (EGF) and fibroblast growth factor (FGF) in enhancing corneal epithelial stratification has been the subject of several studies as early as in the seventies. Gospodarowicz et al. (1977) reported that adult or fetal bovine epithelial cells scraped from whole cornea and cultured as pure epithelial monolayers show a mitogenic response to FGF, but not to EGF. Whole cornea, in vivo or in vitro, however, do respond to EGF by epithelial proliferation and stratification (Frati et al., 1972; Savage and Cohen, 1973; Cohen and Savage, 1974; Gospodarwicz et al., 1977). It therefore seems likely that the corneal epithelium requires the presence of the stroma in order to respond to EGF. Interestingly, Sun and Green (1977) observed a mitogenic effect of EGF on human corneal cells when the cells were grown on a feeder layer of mouse *3T3* cells, which is compatible with the idea that mesenchymal-epithelial interactions are involved. More recently, Song et al. (2001) evaluated the role of EGF and injury on the expression of integrin subunits $\alpha 6$ and $\beta 4$. In their studies, an in vitro wound model was used to evaluate corneal wound repair and cellular migration. Rabbit corneal epithelial cell cultures were serum-starved and injured in the presence or absence of EGF or tyrphostin AG1478, an inhibitor of EGF receptor kinase activity. Repair was monitored morphologically and expression was analyzed using in situ hybridization and immunohistochemistry accompanied by confocal microscopy. The addition of EGF to cell cultures induced a dose-dependent increase integrin $\beta 4$ mRNA expression but the constitutive expression of integrin $\alpha 6$ was several fold greater. In the wounded cultures

there was a rapid change in expression at the edge of the wound that was enhanced with EGF. Moreover, there was an increase in β4 and α6 integrins in migrating cells. Changes in integrin expression were accompanied by a transient increase in activation of the EGF receptor. The addition of tyrphostin inhibited both migration of cells and wound repair, the activation of the EGF receptor and phosphorylation of β4 in the cytoplasm. These data indicate that the activation of the EGF receptor plays a critical role in the regulation of integrin receptors and the mediation of cellular migration.

Recently, much other researchs concentrate on corneal epithelium wound healing, for example Stramer et al., (2003). When the epithelium was prevented from resurfacing the cornea after penetrating keratectomy, expression of fibrotic markers (α-sm actin: smooth muscle actin, and filamentous actin) was considerably reduced. TGF-β2 was determined to be a major substance produced by corneal epithelial cells capable of inducing the fibrotic phenotype. In the intact mouse cornea, TGF-β2 was confined to the uninjured epithelium, but was released into the stroma during fibrotic repair. By contrast, TGF-β1 was never found in the epithelium. When epithelial cells were cultured on a basement membrane-like gel or allowed to deposit their own basement membrane in organotypic culture, TGF-β2 production was reduced. Return of a basement membrane after wounding in vivo correlated with loss of the fibrotic phenotype (penetrating incision or ablation injury to the corneal stroma stimulates a typical fibrotic repair response involving hypercellularity, expression of smooth muscle actin, and deposition of a disorganized extracellular matrix). In the epithelial debridement injury model in which the basement membrane was left intact, TGF-β2 remained confined to the corneal epithelium, consistent with the absence of a fibrotic phenotype (Stramer et al., 2003).

3. 4 Tissues interactions and cornea transdifferentiation.

The best established example of metaplasia is the transformation of embryonic corneal epithelium into epidermis, by dermal grafts under the avian corneal epithelium. Coulombre and Coulombre (1971) removed the lens *in vivo* from the 5-day chick eye and inserted a dermal graft in its place. At this stage, the host cornea has not yet been invaded by keratocytes but is ready for invasion. The grafts consisted of (*a*) 13-day-old foot dermis; (*b*) 5-day-old chick head dermis; and (*c*) 14-day-old mouse flank dermis. In the case of graft *a*, scales formed 12 days after surgery in all cases. In the case of grafts b and c, the invading dermal cells 12 days after surgery induced abnormal arrested feathers in the corneal epithelium.

Likewise, in our laboratory, the differentiation of rabbit corneal epithelium of 12- to 24-day embryos (Ferraris et al., 1994), or even adult central corneal epithelium (Ferraris et al., 2000) were studied after recombination with mouse embryo upper-lip, dorsal, or plantar dermis. The origin of the differentiated structures were identified by Hoechst staining. The results show that in the adult, as well as in the embryo, central corneal epithelium basal cells are able to respond to specific information originating from the embryonic dermis by forming hairy skin or a thick epidermis associated to sweat glands, depending on the type of the associated dermis (Ferraris et al., 2000).

In reverse, can other epithelia be transdifferentiated into a smooth and transparent corneal epithelium? Recently, Nakamura et al. (2003) used an autologous cultured oral mucosal epithelial cell to transplant to in rabbit and human corneal stroma. First, they established transplantable autologous cultivated oral mucosal epithelial sheets in rabbits. The in vitro oral mucosal epithelial sheets showed histological characteristics similar to those of in vivo corneal epithelial sheets; for example, positive keratin 3 expression. The transplanted autologous oral mucosal epithelial sheets resembled corneal epithelium and it achieved the recovery of corneal transparency in rabbits. After that, they transplanted the autologous

cultured oral mucosal epithelial sheet to the patients. These epithelial sheets demonstrated positive keratin 3 and 12 specific to in vivo corneal epithelium, tight junction related proteins and proliferative activity. Apparently, the transplanted allogenic human corneal epithelial sheets existed successfully on the corneal surface, and were quite effective in achieving ocular surface stability in severe ocular surface disorders. A few cases, however, developed immunological reactions or opportunistic infections. The authors propose that cultivated oral mucosal epithelium may become the substitute for corneal epithelium in ocular surface reconstruction for human. However, in their experiments, there is no proof of the origin of the cells.

Another interesting work has been done recently: Huang et al. (2004) discussed the possibility of reconstructing corneal epithelium with skin stem cells. They obtained pieces of human and rabbit skin during operation. Rabbit eye balls were taken, and pieces of corneal stroma without epithelium were prepared. Epidermal stem cells from the rabbit epidermis and human epidermis were cultured in vitro. The human epidermal stem cells of the first to 4th generation were implanted on the rabbit corneal stroma and cultured. Three rabbits underwent autotransplantation of the rabbit epidermal stem cells of the first to 4th generation on the pieces of corneal stroma with the superficial lamina removed and then fed for 100 to 114 days. Another 3 rabbits underwent allotransplantation of the rabbit epidermal stem cells of first to 4th generation on the pieces of corneal stroma with the superficial lamina removed and then fed for 100 days. The allotransplanted rabbit corneas showed congestion since the 9th day, even though histological sections showed the corneas were nor so transparent and the epithelium was nor intact with a lot of lymphocyte infiltration, but since the 3th day of transplantation the transplanted human epithelial cells formed a multilayer epithelium that was positive for AE5 antibody (anti-K3) and K19 monoclonal antibodies. The

autotransplanted corneas remained basically transparent without obvious vascular hyperplasia till the cornea specimens were taken.

4. *Pax6* gene.

4. 1 Introduction.

Pax6 encodes a nuclear transcription factor from the pax family. The Pax6 protein can be separated into distinct domains, an amino teminal paired domain (PD), a glycine-rich hinge region (Gln/Gly), a homedomain (HD) and a carboxy terminal Proline/Serine/Threonine (PST) rich transactivation domain (**Fig. 11. A**). *Pax* genes are defined by the presence of a paired-box, which encodes PD, a highly conserved 128 amino acid DNA binding domain (Callaerts et al., 1997). The PD is organized as two independent subdomains, the amino-terminal PAI and the carboxy-terminal RED which can both bind DNA, either independently or synergistically (Treisman et al., 1991; Czerny et al., 1993). These two motifs are always found together, except in a single Drosophila protein, Eyg, which lacks the PAI domain (Jun et al., 1998). Beside their PD, Pax proteins often contain other conserved domains such as a complete or partial HD, or an octapeptide found between the PD and the HD. The HD is another DNA-binding domain, the specificity of which depends on a crucial residue found at position 50. Most homeoproteins, including all Hox proteins, bear Glutamine (Gln) at this position (Q50) (Macdonald et al., 1996). The HD found in *Pax6* gene is characterized by a Serine at this position, and all Prd-class HDs bearing a S50 are found in Pax proteins. They can bind as homo-or as heterodimers with any paired-class HD to a palindromic DNA sequence (Wilson et al., 1995). The nine human *Pax* genes can be placed into five phylogenetic groups (Sun et al., 1997): (1) *Pax1* and *Pax9*; (2) *Pax3* and *Pax7*; (3) *Pax4* and *Pax6*; (4) *Pax2*, *Pax5* and *Pax8*; (5) cnidaria *PaxA* and Drosophila *Pox-neuro*. *Pax3/7*, as well as *Pax4/6*, contain both a HD and a PD. The Pax proteins are, therefore, multifunctional

transcription factors able to bind to a wide variety of sites either through individual domains, or through cooperative interaction among these domains.

Pax6 is a transcription factor essential for the development of tissues including the eyes, and central nervous system shown here in the chick (**Fig. 11. B-D**), as well as some endocrine gland, of vertebrates and invertebrates. It regulates the expression of a broad range of molecules, including transcription factors, cell adhesion and short-range cell-cell signalling factors, hormones and structural proteins. It has been implicated in number of key biological processes including cell proliferation, migration, adhesion and signalling, both in normal development and in oncogenesis. The mechanisms by which *Pax6* regulates its downstream targets likely involve the use of different splice variants and interactions with multiple proteins, allowing it to generate different effects in different cells (Simpson and Price, 2002).

Pax6 was first isolated from humans, mice and zebrafish (Ton et al., 1991; Puschel, Gruss, and Westerfield, 1992) and subsequently from other vertebrates and invertebrates. A Drosophila gene that contains both a paired box and a homeobox and has extensive sequence homology to the mouse *small eye* gene was isolated and mapped to chromosome IV in a region close to the *eyeless* locus (Quiring et al., 1994). Two spontaneous mutations, *ey2* and *eyR*, contain transposable element insertions into the cloned gene and affect gene expression, particularly in the eye primordia. This indicates that the cloned gene encodes *ey*. The finding that *ey* of Drosophila, *Small eye* of the mouse, and human *Aniridi*a are encoded by homologous genes suggests that eye morphogenesis is under similar genetic control in both vertebrates and insects, in spite of the large differences in eye morphology and mode of development. All these genes in different species are homologous and called now *Pax6*. The human *Pax6* gene was cloned as a positional candidate for the neurodevelopmental disorder aniridia, in which patients commonly manifest one of a number of ocular abnormalities including iris hypoplasia, cataracts, foveal dysplasia, optic nerve hypoplasia and nystagmus.

A number of allelic variants of the *Pax6* gene were subsequently identified in patients suffering from aniridia, Peter's anomaly, keratitis, foveal hypoplasia and ectopia pupillae, implicating the mutant Pax6 protein in the pathogenesis of these eye conditions (Simpson and Price, 2002). Strains of mice carrying mutant *Pax6* gene have a characteristic small eye (Hill et al., 1991), a phenotype analogous to that of human aniridia. *Pax6* is a semidominant mutation, heterozygous mice have abnormal small eye and small nasal cavities, and homozygous mutant have no eyes and no nasal cavities, and no olfactory bulbs (Hogan et al., 1986). Moreover, they show defects in neuronal differentiation and migration that lead to abnormal cortical plate formation (Schmahl et al., 1993). Pax6 protein is thus necessary for the normal development of eyes, nose and brain. Since, like the lens and nasal cavities form from the invagination of an ectodermal placode, these phenotypes have been suggested by Hogan et al. (1986) to result from a failure of the early placode differentiation. By targeted expression of the mouse *Pax6* complementary DNA in various imaginal disc of Drosophila, ectopic eye structures (**Fig. 12. A-B**) were induced on the wings, the legs, and on the antennae (as a review: Gehring, 1995).The ectopic eyes appeared morphologically normal and consisted of groups of fully differentiated ommatidia with a complete set of photoreceptor cells. These results support the proposition that *Pax6* is the master control gene for eye morphogenesis (Halder et al., 1995). Chow et al. (1999) reported that misexpression (overexpression) of *Pax6* in the vertebrate Xenopus laevis led to the formation of some ectopic differentiated eye structures in the head (**Fig. 12. C**). Multiple molecular markers indicated the presence of mature lens fiber cells, ganglion cells, Muller cells, photoreceptors and retinal pigment epithelial cells in a spatial arrangement similar to that of endogenous eyes. However, the overexpression of *Pax6* in chick ectoderm (Kamachi et al., 2001; 2004) was reported to not lead to ectopic eye structure (**Fig. 12. D**). It is only when *Pax6* is cotransfected with *SOX2* that ectopic lens placodes (**Fig. 12. E**) are obtained.

4. 2 Targets of *Pax6* in eye development.

The finding that *Pax6* is not only highly conserved in sequence between vertebrates and invertebrates but also share deep functional similarity has aroused intense interest. In particular, some of the genes that *Pax6* regulates during eye development have been identified in a number of species (Simpson and Price, 2002) and are discussed in the following section. As *Pax6* plays a crucial role in regulating common genetic programmes during eye development that has been conserved during evolution (Walther and Gruss, 1991), it has been suggested that its ancestral role was to generate a structure sensitive to light (Sheng et al., 1997; Pichaud, Treisman and Desplan, 2001).

4. 2-1 *Pax6* and transcription factors.

The Drosophila homeobox-containing gene *sine oculis* (*so*) is directly regulated by *Pax6* through a regulatory element located in the long intron of the *so* gene (Niimi et al., 1999). This gene plays a crucial role in eye development along with at least five other genes encoding nuclear factors, including *eyes absent* (*eya*) and *dachshund* (*dac*) (Kumar and Moses, 2001). Interestingly *Six3*, a member of the *sine oculis* family, is also expressed in vertebrate eye development and may also be under the direct control of *Pax6* in Xenopus (Chow et al., 1999) and mice (Ashery et al., 2000).

Another transcription factor regulated by *Pax6* during vertebrate eye development is *Maf*, a member of the *v-Maf* oncogene family, which plays an important role in the cellular differentiation of several tissues (Blank and Andrews, 1997). In vitro work has indicated that expression of *Maf* is strongly activated by *Pax6* (Sakai et al., 2001). In the developing eye, both *Maf* and *Pax6* are expressed in the region where lens epithelial cells are differentiating to lens fibre cells, suggesting that *Pax6* may indeed directly regulate the expression of *Maf*.

Pax6 also controls the genes of the basic helix-loop-helix (bHLH) transcription factors Mash1, Math5 and Neurogenin2 and has been shown to bind directly to sequences in their enhancers and promoters (Marquardt et al., 2001; Scardigli et al., 2001). These bHLH proteins are known to play important roles in cell determination and differentiation during embryonic development and it is likely that they mediate the effect of *Pax6* on retinal progenitor cell fate (Marquardt et al., 2001). *Prox1*, a member of the Prospero homeobox protein family, may be also directly regulated by *Pax6* in the developing eye (Ashery et al., 2000) and *Pax6* may be able to regulate its own expression (Piaza et al., 1993).

4. 2-2 *Pax6* and crystallins.

The crystallins, which make up 80-90% of the soluble protein of the lens, are coded by the most numerous set of genes known to be targets of *Pax6*. Many crystallin proteins have two functions, both as components of the transparent refractive lens matrix and also as either chaperonins, metabolic enzymes or heatshock proteins (Piatigorsky, 1998). The *αA-crystallin* gene is expressed almost exclusively in the lens where it codes up approximately to 25% of the total water soluble protein, whereas *αB-crystallin* is expressed in the lens, but also in the skeletal muscle, heart and lung (Horwitz, 1992; Kantorow and Piatigorsky, 1994). Expression and electrophoretic mobility shift assays (EMSA) studies have demonstrated that there is a Pax6-binding site in the promoter of *αA-crystallin* that functions in concert with a cyclic-AMP response binding (CREB) element (Cvekl et al., 1995). The *β-crystallin* gene appears to be repressed by *Pax6*. Cotransfection of a plasmid containing the *βB1-crystallin* promoter fused to the *chloramphenicol acetyltransferase reporter* gene and a plasmid containing the full-length mouse *Pax-6* coding sequences into primary lens epithelial cells of chick embryo led to >90% reduction in reporter gene expression driven from the *β-crystallin* promoter

(Duncan et al., 1998). In this experimental system, deletion of the C-terminal transactivation domain of *Pax6* in the expression construct did not ablate repression, suggesting a critical role for the paired and/ or homeodomains. The *δ1* and *δ2-crystallin* genes are taxon specific in that they are restricted to birds and reptiles. The *δ1* gene is predominantly expressed in the lens and has two binding sites for *Pax6* in its third intron (Cvekl et al., 1995). Moreover, *Pax6* cooperates with *SOX2*, which is one of other transcription factors expressed in the eye, for the overexpression of *δ-1 crystallin*. *Pax6* is widely expressed in the head ectoderm before the lens is formed. Once the optic vesicle makes contact with the head ectoderm, the *SOX2* and *SOX3* genes (in chickens; only *SOX2* in mice) are activated in the ectoderm only in the region of contact, and they immediately initiate synthesis of the encoded proteins (Kamachi et al. 1998). Thus, in embryonic lens development, the induction of *SOX2* (and *SOX3*) in the *Pax6*-expressing head ectoderm allows SOX2/3 proteins to meet Pax6 in the same cell nucleus (Kamachi et al., 2001). To confirm the combined action of SOX2 and Pax6 in the lens development, Kamachi et al. (2001) performed studies by using electroporation in the lateral head ectoderm of the chicken embryo at stage 10 (48 hours of incubation) immediately before the occurrence of the lens induction. With *Pax6* vector alone, they observed no ectopic crystallin expression (**Fig. 12. D**), but upon electroporation of *SOX2* and *Pax6* together, cell clusters expressing *δ-1 crystallin* developed in the ectodermal surface outside but next to the eye (**Fig. 12. E**), and these cell clusters had the characteristics of the lens placode: a thickened epithelia structure with expression of placode markers in addition to *δ-1 crystallin*.

4. 2-3 *Pax6* and keratins.

Keratins contribute to intermediate filaments and are expressed by many different types of epithelial cell (see page 20). Expression of the keratin pair keratin12 / keratin 3 was believed to be restricted to corneal epithelium (Sun et al., 1983). Recently, in cotransfection

experiments, *Pax6* has been shown to upregulate reporter gene expression from a *keratin-12* promoter driven reporter construct in human corneal epithelial cells (Liu et al., 1999).

4. 3 Functions of *Pax6* during eye development.

Pax6 is expressed in the anterior neuroectoderm before optic vesicle formation in fish (Krauss et al., 1991; Lossli et al., 1998), frog (Hirsch and Harris, 1997), and chick (Li et al., 1994). In vertebrates, the function of *Pax6* in eye development has been revealed by the mouse *Pax6* null mutant. In homozygotes, eye development arrests after formation of the optic vesicle but before lens placode induction, moreover the small optic vesicles degenerate during subsequent development (Hogan et al., 1986; Hill et al., 1991). In addition, tissue-specific gene targeting and embryologic experiments reveal that *Pax6* functions autonomously in the prospective lens ectoderm for lens placode formation. Van Raamsdonk and Tilghman (2000) have studied lens development in $Pax6^{Sey\text{-}1Neu/+}$ embryos to understand the basis of the haploinsufficiency. The formation of the lens pre-placode appears to be unaffected in heterozygotes, as deduced from the number of cells, the mitotic index, the amount of apoptosis and the expression of *SOX2* and *Pax6* in the pre-placode. However, the formation of the lens placode is delayed. The cells at the edge of the lens cup fail to express N-cadherin, undergo apoptosis, and the lens fails to detach completely from the ectoderm. All together, these studies indicate that, during vertebrate oculogenesis, the transition from pre-placodal ectoderm to lens placode is one of the earliest developmental steps for which *Pax6* is required.

Mice chimeras were used to study roles of *Pax6* during complex tissue-tissue interactions and in tissues that do not form in the mutants (Collinson et al., 2000; Quinn et al., 1996). Production of chimeras allows fine-scale analysis of autonomy and non-autonomy of gene function at the single cell level (Rossant and Spence, 1998; West, 1999) and is

especially useful to study tissues where promoters that efficiently allow expression of Cre recombinase have not been identified. By making *Pax6*$^{+/+}$⇔*Pax6*$^{-/-}$ chimeras that were a mixture of wild-type and *Pax6*$^{-/-}$ cells, Quinn et al. (1996) showed that in chimeras that were composed of less than 50% *Pax6*$^{-/-}$ cells ("low-percentage chimeras"), invaginated nasal epithelia were formed and eyes developed that had an abnormal, two-layered retina (an inner layer that is the prospective neural retina and outer layer that will become the retinal pigmented epithelium), and cornea. Analysis of the distribution of *Pax6*$^{-/-}$ cells in such chimeras at 12.5 days of gestation showed that mutant cells could not contribute to the lens or nasal epithelium. Mutant cells were present at extremely low frequency in the neural retina and could contribute to outer layer of the optic cup, the presumptive retinal pigmented epithelium, but did not form pigment. *Pax6*$^{-/-}$ cells contributed only poorly to the neural retina, forming small clumps of cells that were normally restricted to the ganglion cell layer at 16.5 days of gestation. The segregation and near-exclusion of mutant cells from the nasal epithelium mirrored the behaviour of mutant cells in other developmental contexts, particularly the lens, suggesting that common primary defects may be responsible for diverse *Pax6*-related phenotypes. Collinson et al. (2000) showed that the near-exclusion of mutant cells from the future lens and retina was manifest at 9.5 of gestation, and that *Pax6* is required for maintainance of lens competence and for the adhesion of the optic vesicle to the prospective lens placode. The study did not address whether there is an autonomous requirement for *Pax6* at or before nasal

placode formation. Then in another work by Collinson et al. (2003), it was found that between embryonic days 10.5 and 16.5, *Pax6* is autonomously required for cells to contribute fully not only to the corneal epithelium, where *Pax6* is expressed at high levels, but also to the corneal stroma and endothelium, where the protein is detected at very low levels.

In addition, *Pax6* is observed to be upregulated in repairing corneal epithelium of wound healing (Sivak et al., 2000).

4. 4 Develomental function of *Pax6* in other sites.

Pax6 is expressed not only in the developing eyes and nasal cavities, but also at many other neural and non-neural sites. In mammals, it is expressed in pancreas, gut, pituitary, brain and spinal cord from the early stages of embryonic development (Walther and Gruss, 1991). Several mutant alleles of *Pax6* exist in mice, the most commonly studied of which are the Pax^{Sey} and $Pax6^{Sey\text{-}Neu}$ alleles and these have similar phenotypes (Hogan et al., 1986; Schmahl et al., 1993). Both alleles harbour mutants that encode premature stop-codons resulting in truncation of the Pax6 protein. The $Pax6^{tm1pgr}$ allele is a targeted mutation in which the *Pax6*-coding sequence has been disrupted (StOnge et al., 1997). Homozygous $Pax6^{Sey}$, $Pax6^{Sey\text{-}Neu}$ and $Pax6^{tm1pgr}$ mutants lack eyes and nasal structures and die at birth with severe abnormalities of the central nervous system (CNS), including patterning and growth defects in the developing forebrain (Hogan et al., 1986; Schmahl et al., 1993; Quinn, West and Hill, 1996; Stoykova et al., 1996; Caric et al., 1997; Mastick et al., 1997; Warren and Price, 1997; Gotz, stoykova and Gruss; 1998; Pratt et al., 2000). To understand the functions of *Pax6* in these regions, it is essential to identify its downstream target genes. Many studies have suggested potential targets for *Pax6* on the basis of changes in the expression of genes in mice lacking functional *Pax6* but, such changes may be indirect effects via primary changes in one or more intermediate genes. On the one hand, however, some genes whose expression has been proposed as being influenced by *Pax6* are the same as those shown to be directly regulated by *Pax6* in the developing eye. On the other hand, some genes that are thought to be directly regulated by *Pax6* such as those encoding hormones, are not expressed in the eye. It is likely that a full description of the functions of *Pax6* will eventually need to include an

explanation not only of how it controls its generally distributed downstream genes, but also how it regulates some genes in only one or a few tissues (Simpson and Price, 2002).

BUTS DE LA THESE

Plusieurs questions sont soulevées à propos de l'étude de la différenciation de la cornéen. Elles peuvent se résumer comme suit : quels sont les rôles respectifs du master gene Pax6 qui est exprimé dans tous les tissus de l'œil et des signaux qui peuvent être transmis à l'épithélium de cornée présomptive par son environnement? Ce dernier comprend le cristallin et le mésenchyme d'origine des crêtes neurales qui migre sous l'ectoderme cornéen et constitue le stroma.

Nous avons choisi comme modèle principal l'embryon de poulet d'un accés facile pour réaliser soit la microchirurgie à un stade précoce soit l'électroporation in vivo. Notre modèle secondaire a été l'embryon de lapin et de souris avec lesquels dès que la cornée est constituée on peut comme avec le poulet réaliser des recombinaisons épithélio-mésenchymateuses, et qui, en tant que mammifères, sont plus proches du modèle humain.

Le travail préliminaire à cette étude a été d'établir le profil de distribution d'expression des gènes Pax6 et K12 durant le développement de la tête.

La première question était de savoir si l'expression accrue de Pax6 était capable de promouvoir, comme chez les amphibiens (Chow et al., 1999) la formation de tissus oculaires dans l'ectoderme de la tête. Après le commencement de ma thèse, alors que mes expériences d'électroporation de Pax6 ne donnait que des résultats négatifs, un autre groupe (Kamachi et al., 2001) montrait qu'effectivement, cela n'était pas le cas.

La seconde question concernait le rôle potentiel du cristallin dans l'induction de la cornée. En effet, on croyait généralement (pour une revue voir Hay, 1980) et cela est repris actuellement sur internet, que la vésicule cristallinienne induisait l'ectoderme sus-jacent à se différencier en épithélium cornéen. Ce dogme était-il exact? Alternativement, le cristallin, connu à présent (Lovicu et Overbeek, 1998) pour produire de grandes quantités de FGF10,

était-il seulement requis pour promouvoir la croissance des tissus oculaires? Dans ce cas, le contact à 2.5 jours d'incubation chez le poulet entre la vésicule optique et l'ectoderme sus-jacent est-il responsable de l'induction pratiquement simultanée du cristallin et de l'épithélium cornéen? Nous nous sommes aussi demandé si la vésicule cristallinienne était nécessaire à la migration des fibroblastes périoculaires provenant des crêtes neurales?

La troisième question était d'établir quel pouvait être le rôle du mésenchyme, soit le stroma cornéen, soit le stroma limbal, lors de la différenciation de l'épithélium cornéen. En effet, il est bien connu que chez l'embryon (Dhouailly, 1977) et aussi chez l'adulte (coulomb et al., 1998) dans le cas du psoriasis, que le derme est responsable de la morphogenèse de l'épiderme et de la formation des phanères (Dhouailly, 1977 ; Jahoda et Reynolds, 1996). De même, le derme est responsable de la synthèse de la kératine K9, qui est une kérartine exprimée uniquement dans la région plantaire (Delorme et Dhouailly, 1989 ; Yamaguchi et al., 1999).

En ce qui concerne la cornée, des travaux précédents de notre laboratoire ont montré que chez le lapin, l'épithélium cornéen de l'embyon (Ferraris et al., 1994) et même de l'adulte (Ferraris et al., 2000) est capable de donner naissance à un épiderme s'il est associé à un derme embryonnaire. En collaboration avec le Dr. D. Pearton, postdoctorant dans notre laboratoire, j'ai étudié l'expression de Pax6 et de K12 dans les recombinaisons d'épithélium de cornée de lapin adulte et de derme embryonnaire de souris (Pearton, Yang et Dhouailly, PNAS, sous presse). En ce qui concerne l'épithélium cornéen de poulet, des expériences précédentes (Coulombre et Coulombre, 1971 ; Zak et Linsenmayer, 1985) avancaient que cet épithélium pouvait être transformé en épiderme porteur de plumes, mais seulement avant 7 jours d'incubation. J'ai répété ces expériences chez le poulet et suivi l'expression de Pax6 et de K12.

Comme l'expression de Pax6 et celle de K12 ne sont pas maintenues dans l'assise basale quand l'épithélium cornéen de lapin adulte est associé au derme embryonnaire, ceci pose la question de savoir si le stroma cornéen ou le stroma limbal, chez l'embryon et chez l'adulte jouent un rôle d'induction ou de maintien lors de l'expression de ces deux gènes. Une alternative est que l'expression de Pax6 et de K12 sont activées de façon autonome et que l'association de l'épithélium cornéen avec un derme embryonnaire provoquerait leur inhibition.

Une autre question concerne la possibilité ou non de transdifférenciation de différents épithéliums en épithélium cornéen. Cette question est très improtante pour la recherche clinique. J'ai étudié cette question tout d'abord chez le poulet, puis chez le lapin. Les derniers résultats chez les mammifères sont en cours d'analyse et seront présentés à la soutenance orale.

AIMS OF THE THESIS.

The aims of my thesis were to study tissue interactions and genes involved in differentiation and transdifferentiation of corneal epithelium. There are several questions which arise about the mechanisms involved. They can be summarized as follows: what are the respective roles of *Pax6*, the master gene which is expressed in all eye tissues and of signalling factors that can be transmitted to the presumptive corneal epithelium by tissues of its environment, i.e. the lens vesicle in the early embryo, then the corneal stroma. We choose the chick embryo as a model because of its suitability both for microsurgery at early stages of organogenesis and localized transgenesis by in ovo electroporation. Our second model was the rabbit embryo which is as suitable as the chick to perform heterotopic epithelial/mesenchymal recombinants and more close to the human model.

The preliminary work to this study was to establish the pattern of expression of *Pax6* and *K12* during development of the head.

The first question was whether the ectopic overexpression of *Pax6* is able to promote, as in the amphibians (Chow et al., 1999), the formation of eye tissues in the avian head. After the beginning of my thesis, Kamachi et al. (2001) showed that is was not the case, and our results are in conformity.

The second question concerned the putative role of the lens in cornea induction. Indeed, it is generally thought (Hay, 1980) that the lens vesicle induces its overlying ectoderm to become the corneal epithelium. Is this dogma true? Or is the lens, that is known to produce in large amounts FGFs (Lovicu and Overbeek, 1998), only required for the general growth of the eye? Does the contact between the optic vesicle and the ectoderm induces both the lens and corneal ectodermal territories at almost the same time? Is the lens required for the migration of mesenchymal cells of neural crest origin to form the corneal stroma?

The third question was to establish whether the mesenchyme, i.e. the corneal or the limbal stroma has a role in corneal epithelium differentiation. Indeed, the dermal component of skin was shown to be responsible in the embryo not only for the induction of hair follicles, but also for the number of epidermal strata, that changes according to the body region (Dhouailly, 1977), and also in the adult, in the case of psoriatic disease (Coulomb et al., 1998). Likewise the plantar dermis is responsible for the synthesis of K9, which is specific for plantar epidermis (Delorme and Dhouailly, 1989; Yamaguchi et al., 1999).

In what concerns cornea, previous studies in our laboratory showed that in rabbit, the corneal epithelium of the embryo (Ferraris et al., 1994) or even of the adult (Ferraris et al., 2000) is not able to keep its characteristics and is transformed into an epidermis when it is associated to an an embryonic dermis. In collaboration with Dr. D. Pearton, a postdoctoral fellow in our laboratory, I studied the expression of Pax6 and K12 in such heteropic recombinations involving a rabbit corneal epithelium (Pearton, Yang and Dhouailly, submitted). In what concerns the avian corneal epithelium, some ancient experiments (Coulombre and Coulombre, 1971; Zak and Linsenmayer, 1985) suggested that it can be transformed into an epidermis with feathers, but only before 7 days of embryonic development. I repeated those experiments in order to confirm them or not by following the expression of Pax6 and K12.

As Pax6 expression was not maintained in the basal layer when the adult rabbit corneal epithelium was associated to a dermis, the question was whether the embryonic corneal stroma, or a limbal stroma in the adult, are able to trigger or maintain *Pax6* and subsequent *K12* expression in different epithelia? Alternatively, are these expressions automously activated in corneal epithelium, and inhibited by its association to a dermis? Is the regional origin of the epithelium important? In other words, does the expression of *Pax6* is required by the putative epithelium? In brief, are different epithelia able to transdifferentiate into a corneal

epithelium? This is the most important for targeting the treatment in clinic because there are no sufficient donnor corneas to graft to the patients for the cornea diseases such as cornea dystrophy, cornea degeneration, keratoconus, acidic and basic burning, and so on. I studied first this question using chick tissues, then rabbit tissues. During the course of this thesis, some results by a Japanese group (Nakamura et al., 2003) and a Chinese group (Huang et al., 2004) are the first indication of such a possibility in human and rabbit. I present in this manuscript the results that I obtained in chick and will be able at the oral to present the results obtained in rabbit.

RESULTATS

En français je ne prèsenterai ci-après que mes principaux résultats et sous forme résumée.

I. Profil d'expression des gènes Pax6 et K12 dans les épithéliums céphaliques.

Chez le poulet l'expression de Pax6 (proteine nucléaire) est détectée à l'aide d'un anticorps polyclonal (Eurogentec, France) à 4 et 7 jours d'incubation dans la cupule optique, la vésicule cristalllinienne et l'ectoderme sus-jacent, ainsi que dans la placode puis la cavité nasale et l'épithélium de la cavité orale. A partir de 7 jours l'expression de Pax6 décroit dans les épithéliums nasal et oral et n'est plus très rapidement détectée. Par contre l'expression de Pax6 continue dans les structures oculaires et en particulier l'épithélium cornéen. La kératine K12 (detectée à l'aide d'un anticorps monoclonal réalisé au laboratoire) commence à être exprimée à 12 jours d'incubation et est fortement exprimée à partir de 14 jours d'incubation dans l'épithélium cornéen.

De plus cette expression est présente seulement dans l'épithélium cornéen. De même, chez l'embryon de lapin, K12 est exprimée seulement dans l'épithélium cornéen. En ce qui concerne Pax6, l'anticorps polyclonal ne pouvait être utilisé.

J'ai disposé de coupes de cornée humaine adulte et de coupes d'épithélium oral d'humain de 3ans et adulte. Pax6 et K12 sont exprimés dans la cornée mais pas dans l'épithélium oral.

II. Effets de l'expression de Pax6 par électroporation dans la tête de l'embryon de poulet.

J'ai utilisé une construction Pax6 DP et une construction Pax6 DN (donnés par le Dr. Nakamura). Dans les deux cas je les ai mélangés à une construction GFP afin de contrôler l'efficacité de mon électroporation.

Je n'ai obtenu aucun survivant en réalisant les électroporations au stade plaque neurale (24 heures d'incubation). Les électroporations réalisées à 2.5 jours ont donné les résultats suivants. L'utilisation de Pax6 dominant positif a conduit au non établissement correct de l'axe dorso-ventral de l'œil : la fissre choroidienne, normalement présente du côté ventral se retrouve en position dorsale et/ou dupiquée une ou deux fois.

Aucune structure oculaire ectopique n'a été obtenue.

L'électroporation de Pax6 dominant négatif a conduit à de nombreuses malformations : œil absent ou de petite taille, bec croisé, cerveau faisant protrusion.

III. Le cristallin n'est pas requis ni pour l'induction de l'épithélium cornéen ni pour la migration des fibroblastes du stroma. Expériences réaliseés chez le poulet.

La vésicule cristallinienne a été otée au moment où elle se détache de l'ectoderme (2.5 jours d'incubation) ou plus tardirement, à 5 jours. Dans tous les cas, les mêmes résultats ont été obtenus. L'œil anormal qui se développe est de petite taille. Cependant les fibroblastes du mésenchyme péri-oculaire migrent précocément sous l'ectoderme qui exprime à 14 jours d'incubation la kératine K12. Ce mésenchyme joue-t-il un rôle dans la différenciation de l'épithélium cornéen?

IV. Capacités de différenciation de l'épithélium cornéen.

Les associations d'épithélium cornéen de lapin adulte et de derme embryonnaire de souris provoquent l'extinction de l'expression de Pax6 et de K12 dans les cellules épithéliales de la couche basale et la formation ultérieure de follicule pileux.

Par contre, lorsque j'ai répété ce type de recombinaison chez le poulet, je n'ai obtenu l'extinction de Pax6 et la formation de plumes que lorsque le derme embryonnaire de poulet ou de caille est inséré sous l'ectoderme à 3.5 jours d'incubation. Les recombinants comportant un épithélium cornéen de 5 jours ne montrent que par endroit la répression de l'expression de Pax6 et de K12 et ne forment jamais de plumes. L'épithélium cornéen recombiné à 7 jours d'incubation n'est aucunement perturbé dans sa différenciation, expression de Pax6, puis de K12.

V. La transdifférenciation en épithélium cornéen est-elle possible? Expérimentations réalisées chez le poulet.

Lorsque l'épithélium nasal, oral, ou cutané embryonnaires sont recombinés stroma cotnéen ou limbal, ils continuent à se différencier en conformité avec leur origine, même dans le cas d'épithélium cutané électroporé avec la construction Pax6 DP.

RESULTS.

I. Head expression pattern of Pax6 and K12.

I. 1 Introduction.

Ours aims are to know whether Pax6 and K12 expression would be detected in the epithelia of the developing embryo head. To study the pattern of expression of Pax6 and K12 proteins, we choose the chick and rabbit embryo as models because of the knowledge what we have already about keratin expression in their corneal epithelium (Chaloin-Dufau et al., 1990). For the chick embryo, I was able to recover the embryos, fix and section them from stage day 4 to newborn. For the rabbit embryo, it was not so easy to get all the stages of them. So I chose two stages: 20 days of gestation and newborn. For the human, because we could not get the human embryo, we use human adult cornea and mouth tissue for comparison.

I. 2 Materials and methods.

Isa brown fertilized eggs were obtained from a single commercial source (SFPA, St Marcellin, France). Rabbit embryos were from Elevage Scientifique des Dombes (Vif, France). Eggs were incubated at 38° ± 1° C at different stages. Human corneas were gotten from the patients after cornea transplantation in the ophthalmology department, and mouth tissue were obtained from the oral surgeries in the plastic surgery department of Grenoble Centre University Hospital (CHU).

Immunohistochemistry was performed on cryosections. Chick and rabbit embryo heads and human cornea and mouth tissues were embedded in Tissue-Tek OCT compound, then frozen at -80° C. Sections (6μm) were fixed in 4% paraformaldehyde or acetone, washed in TBS and blocked in TBS containing 2% normal goat serum and 1.5% bovine serum albumin for 30 minutes at ambient temperature and then processed for single or double

immunofluorescence. I used Pax6 polyclonal antibody (Eurogentec, France) and Pax6 monoclonal antibody (produced in Developmental Studies Hybrioma Bank, University of Iowa, USA; Ericson et al., 1997), AK12 monoclonal antibody (produced in our laboratory) to stain frozen sections of chick eye at different developmental stages. AK12 monoclonal antibody and Pax6 monoclonal antibody (both for 1 hour at room temperature, without dilution), or Pax6 polyclonal antibody (1:1000, overnight at 4°C) as the primary antibody were incubated for different sections. Alexa 488 (Molecular Probes, Oregon) as the secondary antibody (goat antimouse, 1:1000 red or 1:500 green) was applied there after for 30 minutes incubation, then washed with TBS. After secondary antibody, sections were stained with Hoechst 33258 or DAPI, rinsed in pure water, dehydrated and mounted.

I. 3 Results: Chick.

From 4 days of development, the main structures of the eye are present. First, Pax6 protein is strongly expressed in the nuclei at day 4 in the overlying ectoderm, which is part of future corneal epithelium, in the lens vesicle and in the retina that develops from neuroderm. At 7 days (**Fig. 13. A**), Pax6 expression is detected in nuclei of corneal epithelium, lens, iris and retina. At 21 days, Pax6 is expressed in corneal epithelium, iris and retina similar as it is expressed at 7 days, particularly in the lens capsule, and is not detected in the lens nucleus and fibers (**Fig. 13. B**).

Appearance of keratin 12 during embryonic development of chick cornea was confirmed as the results obtained by Chaloin-Dufau et al. (1990): K12 is not expressed at 7 days (**Fig. 14. C**) in contrast to Pax6 (**Fig. 14. A**), and its expression which only starts by 12 days of incubation, is clearly detectable at 14 days of incubation in the cytoplasm (**Fig. 14. D**), whereas Pax6 is detected in the nuclei (**Fig. 14. B**).

In the beak, there are two cavernous nasal cavity, that are lined by olfactory concha and respiratory concha, and covered by nasal epithelium (**Fig. 15. A**). Pax6 and AK12 staining was performed from day 7 to 18 of incubation. Pax6 expression is detectable at 7 days of incubation in the developing nasal cavity (data not shown). From 12 to 18 days, the nasal epithelium, which covers the central concha, shows that Pax6 is not detected in the epithelium cells, either nuclei or cytoplasm (**Fig. 15. B-D**). However, the epithelium which is close to the nasal meatus, shows that Pax6 is expressed in the nuclei of the epithelium cells, also in the cytoplasm at 12 days of incubation (**Fig. 15. E**). By day 14, Pax6 expression is downregulated, a weak labelling remaining in the nuclei and cytoplasm of the epithelium (**Fig. 15. F**). At 18 days, Pax6 expression is no more detectable in the nuclei or cytoplasm of the epithelium which is close to the nasal meatus (**Fig. 15. G**).

No K12 protein was detectable at 7 days in nasal epithelium. At 12 days of incubation, perhaps there is a very weak labelling in the cytoplasm of the epithelium which is only close to the nasal meatus (data not shown). From 14 to 18 days of incubation, there is no K12 expression detected in the cytoplasm of the nasal epithelium, either in the center or the periphery of the nasal cavity (**Fig. 15. C, D, F, G**).

Observation of Pax6 expression in oral epithelium was performed from 7 to 18 days of incubation. At 7 days, Pax6 is still expressed in the nuclei and cytoplasm of the oral epithelium cells (**Fig. 16. A**). Then, at the different studied stages (12, 14 and 18 days), Pax6 expression was not detectable in both nuclei and cytoplasm of the oral epithelium (**Fig. 16. B, C, D**).

K12 expression was examined from day 7 to 18 days of gestation. There was no K12 expression detected in the cytoplasm of the oral epithelium at any stage (**Fig. 16. B-D**).

We show here that through all the chick embryonic stages Pax6 is expressed in the nuclei of corneal epithelium, lens capsule and retina but seems no more expressed in the lens fiber

cells, which are in their final step of differentiation. Whereas Pax6 is expressed in the nasal placode (Tiffany et al., 2002). We show that this expression is downregulated by 7/14 days. Likewise Pax6 is only weakly expressed at 7 days in the oral epithelium. Moreover, K12 expression that is restricted to the cytoplasm of corneal epithelium cells appears at a relatively late stage of development.

1. 4 Results: Rabbit.

Immunohistochemical staining of frozen corneal sections of 20-day embryo and newborn with Pax6 and AK12 monoclonal antibodies was performed. The Pax6 monoclonal antibody did not work in the rabbit and the Pax6 polyclonal antibody, as made in rabbit, can not be used. Thus we were unable to check for Pax6 expression in rabbit tissues.

AK12 staining is first detected in the peridermal layer of 17-day embryo (data not shown) as already shown (Chaloin-Dufau et al., 1990). At 20 days, K12 is expressed only in suprabasal layer of central corneal epithelium (**Fig. 17. A, B**). In the 23-day embryo, AK12 staining reached the basal layer (Chaloin-Dufau et al., 1990). K12 expression is present throughout all the epithelium strata in the central cornea at birth (32 days) (**Fig. 17. C, D**).

No K12 expression was detected in nasal (**Fig. 17. E**) and oral (**Fig. 17. F**) epithelium in 20 days embryos, as well as in the newborn.

1. 5 Results: Human.

Pax6 is expressed strongly in both of nuclei and cytoplasm of the adult human central corneal epithelium cells (**Fig. 18. A**) and in the cytoplasm of the basal layer of the limbus (**Fig. 18. B**). K12 is expressed in the cytoplasm of all the cell layers in the central corneal epithelium (**Fig. 18. C**) and in the suprabasal layers of the limbus epithelium (**Fig. 18. D**).

Pax6 and K12 expression were not detected in the human cheek oral epithelium, both in 3 years old (**Fig. 18. E**) and adult (**Fig. 18. F**).

1. 6 Conclusion.

In chick embryos, Pax6 is expressed in the nuclei not only in the developing eye tissues, but also in other head epithelia: nasal epithelium and oral epithelium, at the beginning of organogenesis, as it has been preciously shown for mice (Gehring, 1996; Walther and Gruss, 1991). While Pax6 continue to be expressed in corneal epithelium in the adult, it is rapidly downregulated during development until no more expressed in the nasal and oral epithelia as shown here in different species.

In chick and rabbit, K12 is expressed strongly in the cytoplasm of the corneal epithelium from a late embryonic stage, 14 days in chick, 21 days in rabbit, and throughout all the life of the adult. In human, we were unable to analyse foetal situation and only confirm that K12 is expressed in the cytoplasm of the adult corneal epithelium. No matter of the species, chick, rabbit, or human, there are no K12 expression detected in the nasal or oral epithelium.

Usually, the keratin pair K12/K3 is considered to be corneal epithelium specific (Sun et al., 1983; Liu et al., 1999). However, the corneal type K3, but not K12, was recently shown in rabbit adult oral epithelium (Nakamura et al., 2003) together with the Keratin pair K4/ K13 characteristic of the esophagal epithelium and oral epithelium (Sun et al., 1983). In my observations, no K12 was detected in other head epithelium than cornea, both in chick and mammals, thus K12, but not K3 appears to be corneal specific.

II. Ectopic expression of Pax6 in the head.

II. 1 Introduction.

In order to study the role of an overexpression or a repression of *Pax6* during eye development, we used chick embryo, a model system which allowed to easily perform localized transgenesis in ovo. We used a *Pax6* dominant positive construct containing the VP16 transcriptional activation domain (*Pax6-Pos*) and a Pax6 dominant negative construct linked to the Engrailed repressor dominant (*Pax6-Neg*) (kind gifts of Dr. Nakamura), in electroporation experiments. To control the method, green fluorescent protein (*GFP*) plasmid was mixed with *Pax6-Pos* plasmid together, the first one being easily observed with fluorescent light the day after.

II. 2 Material and methods.

Eggs were incubated at 38° C on their long side, and kept in the same position until when I performed microsurgery. A small hole was made from the opposite side of the air chamber side of the egg in order to take off 4 ml albumin, then the hole was closed with paraffin. Another big hole (diameter around 2cm) from the top of the eggs was covered by tape after opening, and the eggs were returned to the incubator. I used chick embryos at two different stages for *Pax6 dominant positive cDNA* electroporation series. In the first group, embryos at very early stage, HH 7 (24 hours, Hamburger and Hamilton, 1951) were performed operation. Because the embryos are very fragile to dehydratation at this stage, I add a drop of PBS to cover the blastoderm. Then I placed the negative electrode to the left side of the embryo, positive electrode to the right side, and at the same time I pushed vitelline membrane to make the electrodes as the same level as embryo future head ectoderm. Ten volts and 5 sec lasting

pusles were applied because of the diverting electrodes. Unfortunately, I was not able to recover any surviving embryo in this series. For the second group, I used 2.5-day embryos (stage HH 15). I opened the vitteline membrane, and injected mixed plasmid (*Pax6* and *GFP*) in the amniotic cavity. In order to electroporate the left eye, two electrodes were placed on each side of the embryo head (**Fig. 19. A**), the positive electrod on the top of the embryo, between amnion and right optic vesicle, the negative electrod below the embryo, at the level of the left optic vesicle. Then 20 volts and 10 sec lasting pulses were applied. Some of the embryos were recovered the day after electroporation for checking the expression of GFP (**Fig. 19. B**). Most embryos were recovered at 8 and 14 days of incubation. For each embryo, the right developing eye may serve as a control, the best control being that of an untreated embryo of the same series. For the third group, in order to electroporate the cranial ectoderm, two electrodes were placed on ventral and dorsal sides of the head of stage HH 21 embryos (3.5 days), negative electrode on the dorsal side, positive electrodes on the ventral side. The same volt and lasting pusles were applied. The embryos were recovered at 8 and 14 days of incubation.

For the *Pax6 dominant negative cDNA* electroporation series of the left eye at 2.5 days of incubation, the method was the same as that used in the second group of *Pax6 dominant cDNA* electroporation.

II. 3 Results: Effects of *Pax6 dominant positive cDNA* expression.

In order to know whether *Pax6-Pos* plasmid is transferred to the embryo eye or head, I chose 4 cases to recover the day after electroporation in every group. There were two to three cases GFP positive (**Fig. 19. B**).

In the first group, 2 to 5 days after *Pax6 dominant positive electroporation* at stage HH 7, there was no survivor among the 45 electroporated embryos.

For the second group, I was able to recover 18 embryos out of 30 electroporated. In the control eye at day 8 of incubation, there is only one choriod fissure in the inferior, ventral side of the eye (**Fig. 20. A**). Among the electroporated group, 8 cases showed a left eye with an enlarged and mis-orientated choriod fissure (**Fig. 20. B**), and even in 3 cases 2 or 3 choriod fissures (**Fig. 20. C**) in a dorsal position. The dorsal position of the enlarged or multiplied choroid fissures demonstrates an abnormal orientation of the eye.

For the third group (electroporation of the cranial ectoderm), a total of 43 embryos from 80 electroporated embryos were recovered. It should be noted that there was no formation of ectopic eye structures in the head ectodem but some enlarged brain (**Fig. 20. D**). Embryos showed similar results at 8 days and at 14 days of incubation. Moreover, in 7 cases, 2 or 3 choriod fissures, and in 11 cases an enlarged and mis-orientated choriod fissure, were observed.

II. 4 Results: Effects of *Pax6 dominant negative cDNA* expression.

For checking GFP expression the day after *Pax6-Neg* plasmid electroporation, 4 cases were recovered, 3 cases were positive for GFP.

Among 60 electroporated embryos, 8 were recovered at 8 days and 33 at 14 days of incubation (**Fig. 20. E-H**). The total aliving number was 41, and there were 24 cases without left eye and 5 cases with a small abnormal left eye. The beaks in all the cases were crossed, and in some cases the brain extruded from the skull. From all these experiments, electroporation with *Pax6-Neg* (*EnR*) shows a range of effects ranging from no left eye formation (40-55% of the cases) to small or abnormally formed eyes (around 7-8%).

II. 5 Conclusion.

The electroporation of *Pax6-Neg* impeded the formation of eye structures, whereas the electroporation of *Pax6-Pos* led to disruption of the normal dorsal-ventral patterning of the eye. The choroid fissure, which is normally localized in the ventral region of the retina was mislocalized to the dorsal region and/or duplicated one to three times. Moreover this choroid fissure can be enlarged.

In contrast to what happens in Drosophila and Xenopus (see **Introduction 4**, page 32), we confirm the results from Kamachi et al. (2001, 2004) that there is no supplementary eye structures formed after transfection with *Pax6-Pos*, even in the head ectoderm. It should be noted that in the Drosophila experiments of Halder et al. (1995), *Pax6* gene targeted various imaginal discs, which are formed by groups of undifferentiated cells. Likewise, the experiments on Xenopus (Chow et al., 1999; Onuma et al., 2002) were done at 2- and 16-cell stages, which means before the blastocyst stage. We tried to electroporate the anterior region at stage HH 7 (blastoderm stage, i.e. equivalent stage to the amphibian blastocyst), but all the embryo died during the next days. We only were able to recover chick embryos which were electroporated at 2.5 to 3.5 days (respectively stage HH 15-21) chick embryos, which correspond to a relatively advanced stage of embryogenesis, the eyes being in formation.

Thus, ours results emphasize the necessity for a tight control of *Pax6* activity during development as both increased and lowered *Pax6* activity has deleterious effects on eye development. They suggest that a precise amount and distribution of Pax6 transcripts are required for the normal dorsal/ventral orientation of the eye. The co-transfection of *Pax6* and *SOX2* was shown during the course of this thesis to lead to the formation of ectodermal cells expressing δ-1 cristallin (Kamachi et al., 2001; 2004). As Pax6 expression is not enough,

other factors might be required for corneal epithelium commitment during embryonic development and during adulthood upholding.

III. The lens is not required either for corneal epithelium induction and stroma migration.

III. 1 Introduction.

The cornea of all vertebrates begins its development in the ectoderm overlying the lens. In order to study whether the lens is necessary to induction and development for corneal epithelium and stroma migration, the lens vesicle was removed from chick embryo eye at 2.5 to 5 days of incubation, stage HH 15 to HH 25 (Hamburger and Hamilton, 1951). We followed the development of chick cornea between 5 and 14 days of incubation in order to make a comparison between the right operated eye and the left normal eye.

III. 2 Materials and methods.

The eggs were incubated and opened as described in **Chapter II** (page 49). I chose the right eye for operation because it is on the top of the turned embryo head at this stage and it is not very difficult to perform surgery. I made a small incision from the temporal side of the optic vesicle with a ophthalmology microsurgery 30° knife and took off the lens vesicle which was just detached from the ectoderm at stage HH 15-18 (2.5-3 days) (**Fig. 21. A, B**), or the differentiating lens at stage HH 22-25 (4-5 days) from the incision with very sharp forceps. I droped some PBS again, covered the window made in the egg shell with tape and returned the eggs in the incubator for ten days.

I performed immunofluroscent staining as described in **Chapter I (**page 44**)**, by using AK12 monoclonal antibody made in our laboratory (Chaloin-Dufau et al., 1990).

For histology staining, tissues were fixed by 4% paraformaldehyde overnight, rinsed, dehydrated, embedded in paraffin and sectioned at 7μm. The slides were stained with Hematoxylin.

III. 3 Results: Comparison of corneal development, in presence or absence of the lens.

At 2.5 days of incubation, in the unoperated eye, the lens vesicle has just detached from the ectoderm. The lens placode invaginates, rounds up and contacts the new overlying ectoderm. There is still no mesenchymal cells between the ectoderm and the lens (**Fig. 21. C**). At this stage, the corneal epithelium corresponds to the ectoderm which reunites after the lens vesicle has separated from it. After the lens removing (**Fig. 21. E**), in one hour the retina contracts as a strut. Wound healing of the ectoderm recovers the optic vesicle in a few hours. In normal eye, the development of the chick cornea is charaterized by the formation of an acellular stroma under the ectoderm. In the unoperated eye, after 2.5 days (thus at 5 days of incubation) the first fibroblast cell migration forming the endothelium under the acellular primary stroma is almost complete (**Fig. 22. A, B**). At this stage, the corneal epithelium consists of two cellular layers, the periderm and the ectoderm proper. At 7 days of incubation, a second wave of fibroblasts migration have occupied all layers of the stroma and the anterior cornea has formed (**Fig. 22. C, D**). In the operated eye, in contrast, 2.5 days after the surgery (thus at 5 days of incubation), the mesenchymal cells have already migrated and colonized the space under the ectoderm of the operated eye (**Fig. 22. E, F**). At 7 days of incubation, the cornea stroma is thicker than normal, and no endothelium formed, some part of the retina folds without the support of lens and iris and the anterior corner was not formed (**Fig. 22. G, H**). In all 16 cases, the operated eye develops abnormally. The general size is much smaller (**Fig. 21. F**) than the control eye (**Fig. 21. D**). The retina folds, the cells in the retina are not

arranged in order as usual, vitreous body becomes opaque, the anterior chamber is very narrow and the anterior chamber corner closes.Taking off the differentiating lens at stage HH 22-25 (4-5 days), leads to the similar results (22 cases, data not shown).

In what concerns K12 expression, it is clearly detectable in corneal epithelium at 14 days of incubation in the control eye (**Fig. 23. A, B**), and it weakly detectable in the corner of the eyelids (the fornix) (**Fig. 23. A**). I recovered all the operated embryos at 14 days of incubation. In the first group (lens vesicle ablated at stage HH 15-16, 2.5 days), among 86 embryos, I was able to recover 15 survivors. In all of them (100%), K12 expression was present in the corneal epithelium and also in the fornix (**Fig. 23. C-D**). In the second group, (lens ablated at stage HH 18, 3 days), there were 6 survivors among 34 embryos, and also in 100% of cases, K12 was expressed in the corneal epithelium. In the third group, operating at stage HH 22 (4 days), it remained 5 survivors among 25 embryos. In 4 cases, K12 expression was positive (80%). In the fourth group, surgery was performed at stage HH 25 (5 days). I was able to recover 5 embryos among 16. In all of the cases, the corneal epithelium expressed K12.

III. 4 Conclusion.

The lens was considered previously as required to development of the cornea by many scientists (Amprino, 1949; Hay and Merier, 1974; Dodson and Hay, 1974; Coulombre and Coulombre, 1964; Zinn, 1970). Hay (1980) noted that during stage HH 18-22, the chick corneal epithelium is closely related to the lens capsule and optic cup, and suggested that both influence its differentiation.

However, from our experiments, it appears that in the operated eye the migration of the mesenchymal cells occurs in one time under the ectoderm as soon as before 5 days of

incubation, and thus the corneal stroma forms 2 days earlier than it in the unoperated eye. In contrast to what was published before, the lens is only required for the general and harmonious growth of the eye, but not for the migration of fibroblasts of neural crest origin of the corneal stroma. In what concern the differentiation (K12 expression) of the corneal epithelium, either the lens is not needed at all or its effect might be effective at the time when the lens vesicle detach from the ectoderm. Our results support the suggestion, that the optic cup, not the lens, has the first inductive effect on the still undifferentiated ectoderm (Meier, 1977). In this point of view, we propose (**Fig. 24. A-B**) that the contact between the optic neural vesicle and the ectoderm might induce concomitantly both the lens and the corneal epithelium (**Fig. 24. A1**), or alternatively, the corneal ectoderm might be specified soon after the lens placode, by the future iris (**Fig. 24. A3**). This region, which will lead in the adult to the limbal mesenchyme formed in the embryo in contact with the iris (see **Fig. 22. D**) might continue to play an important role (niche for the stem cells) in adult corneal differentiation.

IV. Differentiation abilities of corneal epithelium.

IV. 1 Recombinants of adult rabbit corneal epithelium and embryonic mouse dorsal dermis.

IV. 1-1 Introduction.

Previous results in our laboratory showed that embryo (Ferraris et al., 1994), as well adult (Ferraris et al., 2000) rabbit central corneal epithelium basal cells are able to respond to specific information originating from mouse embryonic dermis by forming hairy skin or a thick epidermis associated to sweat glands, depending on the type (dorsal or plantar) of the associated dermis. Following these results, the question was to follow the expression of both K12 and Pax6 in adult corneal epithelium when it is associated to an embryonic back dermis. In order to know the answer, I collaborated with Dr. David Pearton who is a post-doctorant in our laboratory (Pearton, Yang and Dhouailly, submitted).

IV. 1-2 Materials and methods.

OF1 and athymic nude mice were from Iffa-Credo (Lyon, France), rabbits were from Elevage Scientifique des Dombes (Vif, France). All animals were humanely euthanized. During graft surgery, nude mice were anaesthetized by intraperitoneal injection of 0.3µg of valium and 3µg of imalgene per mouse. All animal procedures were performed according to the procedures of the french Animal Protection and Health Ministry, authorization number 04622 to D.D.

Recombinants were performed between rabbit adult central corneal epithelium and mouse embryonic dermis. The rabbit central cornea was recovered and dissected from the

limbus, leaving a 3 mm border of transparent cornea attached to the limbus in order to ensure that no limbal cells were included. The epithelium was dissociated from the underlying stroma by treatment with saturated EDTA in Ca^{2+} and Mg^{2+} free medium. The epidermis and dermis from the upper-lip of 12.5-day embryos or the back of 14.5-day embryos from OF1 mice were surgically removed and dermis and epidermis separated (**Fig. 25. A**) via protease treatment (1.25% trypsin + 2% pancreatine). The corneal epithelium and embryonic dermis were associated on agar medium (**Fig. 25. B**) for 1 hour and were then grafted under the kidney capsule of swiss nude mice (**Fig. 25. C-E**) using the method of Higgins et al. (1989). The grafts were recovered after various time between 1 and 28 days, embedded in OCT and stored at -80°C before cryosectioning for immunofluorescence or immunohistochemistry. We were able to use the Pax6 polyclonal antibody (Eurogentec, France) as the rabbit tissues were restricted to the epithelium of the recombinants.

IV. 1-3 Results: Expression of Pax6 and K12 in the recombined corneal epithelium.

The difference of rabbit and mouse tissues is based on the staining of their nuclei with Hoechst or DAPI (Cunha and Vanderslice, 1984). The rabbit nuclei DNA is homogenously distributed while mouse nuclei have a punctuate appearance. In all the recombinants, the keratinocytes were clearly of rabbit origin.

As only the central corneal epithelium was used in the experiments, all the cells in the recombined epithelium (even the basal cells) initially express the corneal keratins K12 as well as nuclear Pax6. At day 2 after recombination all cell layers of the epithelium still express Pax6 and K12 (**Fig. 26. A**). Subsequently, the cells of the basal layer begin to downregulate and, by day 4 (**Fig. 26. B**), no longer express K12. In addition, in these cells where K12 has been downregulated, there is a distinct cytoplasmic localization and lowered levels of Pax6,

whereas, in the K12 expressing cells of the suprabasal layers Pax6 is still strongly expressed and localized in the nucleus. This downregulation and relocalization of Pax6 continues, and at day 6, Pax6 is downregulated still further in patches in the basal layer (**Fig. 26. C**). By 13 days of grafting, the cells of the newly forming hair follicles are negative both for Pax6 and K12 (**Fig. 26. D**).

IV. 1-4 Conclusion.

The basal layer of the corneal epithelium turning off expression of K12 and Pax6 , then converting to hair and interfollicular epidermis is under the guidance of the embryonic dermis. The follicles, consisting exclusively of cells originating from rabbit corneal cells, have associated dermal papillae derived from the mouse embryonic dermis. Thus we confirm that an embryonic dermis having reached the stage of dermal condensation formation is able to induce the transdifferentiation of central corneal epithelium into hairs and then interfollicular epidermis as already shown (Ferraris, 2000).

The cells of the basal layer of the central corneal epithelium respond signals emanating from the embryonic dermis and revert from a differentiated phenotype where they express Pax6 gene and the corneal type keratins K12. The first step is the down-regulation and redistribution to the cytoplasm of Pax6 in the epithelial cells in contact with the dermis as it occurs in the basal layer of the limbus. Pax6 expression appears to be further downregulated, or even absent in cells forming the hair peg, while in other areas of the basal layer it is localized to the cytoplasm, and in the K12 positive suprabasal layers it remains nuclear. K12 expression is turned off in those cells where Pax6 is repartitioned from the nucleus to the cytoplasm and hence cannot act as a transcriptional activator. Our experiments confirm that keratin K12 is directly regulated by Pax6 (Liu, 1999). Further interesting question is whether

the loss of Pax6 is a result of the loss of signals from the stroma that might maintain its expression in its normal context, or inhibition signals from the dermis, or dedifferentiation occuring together with the re-entry in the cell cycle that leads to the ingrowing hair buds. It should be noted that the formation of an epidermis composed of rabbit cells occurs only after the achievement of hair follicle morphogenesis.

IV. 2 Recombinants of chick corneal epithelium and chick dorsal dermis.

IV. 2-1 Introduction.

Is chick corneal epithelium able to be transformed into a cutaneous-appendage bearing epidermis as the rabbit corneal epithelium, at least at an embryological stage? In order to answer this question, I used chick central corneal epithelium of different embryological stages associated with a chick (or quail) embryonic dorsal dermis. I first performed microsurgery via inserting 7 days chick dorsal dermis under the corneal ectoderm at 3.5 days of incubation. Then I recombined corneal epithelium of 5 days or 7 days with chick 7-day dorsal dermis (or quail 6-day dorsal dermis). In all the cases the dermis was obtained from embryos having reached the stage of formation of dermal condensation.

IV. 2-2 Materials and methods.

Eggs were incubated at 38° ± 1° C. The eggs were opened at 2.5 days according to the method described in **Chapter II** (page 49).

Because of the difficulty to obtain the corneal epithelium before 5 days of incubation, and in order to do recombinants at an early stage, I took off the lens from the ectoderm at stage HH 21 (3.5 days of incubation) and inserted instead a piece of 7-day dorsal dermis. At this stage, the corneal stroma is still not formed (see **Chapter III**, page 55). The dorsal skin of

7-day chick embryos was dissected and the dermis and epidermis separated via protease treatment (1.25% trypsin + 2% pancreatine) during 15 minutes. After insertion of a piece ($1mm^2$) of dermis under the corneal epithelium, the eggs were closed with tape and returned to the incubator until the 14^{th} day. For a second series, the embryos were sequentially recovered from the day after operation until the 8^{th} day following the grafts.

In the case of 5 to 7 days chick embryo corneal epithelium, recombinants were performed with 7-day chick (or 6-day quail) embryonic dermis. Four types of recombination were done by using different stages of chick corneal epithelium and different kinds of dorsal dermis (**Table. 1**). The methods to obtain the epithelia and the dermis as well as to graft the recombinant under the capsule of nude mice kidney were previously described in **Chapter IV.1** (page 58-59) and showed in **Figure 25**. The grafts on the nude mice were recovered after 2-3 weeks.

Immunofluorescent and histology staining were previously described in **Chapter I** (page 44) and **Chapter III** (page 55).

IV. 2-3 Results: The response of the chick corneal epithelium depends on the stage of embryonic development (Table. 1).

By inserting a piece of 7-day chick dorsal dermis after taking off the lens from the eye at stage HH 21 (3.5 days of incubation), feathers (**Fig. 27. A**) grow from the corneal epithelium in 14 cases among 19 survivors recovered at 14 days of incubation (10.5 days after operation) from 60 operated embryos. Moreover, Pax6 was downregulated and no more detectable 10 days after the graft (**Fig. 27. B-E**). Likewise, K12 expression was not detected, in contrast to the left control eye. Thus, the chick embryonic corneal epithelium is able to be transformed in a feathered epidermis at 3.5 days of incubation. In order to precise when Pax6 downregulation occurs, a second series of experiments was performed and the embryos were fixed and the

right eye analysed for Pax6 expression at 1, 2, 3, 4, 6 and 8 days after the graft. The results show that after the corneal epithelium contact with the embryonic dermis, Pax6 expression is rapidly downregulated from the first day after operation (4.5 days of incubation) and no more expressed from the fourth day after operation (7.5 days of incubation) (**Fig. 27. F-H**). At the sixth day after operation (9.5 days of incubation) the feather buds grow in the place which the corneal epithelium contacts with the dermis. By the eight days after the graft (11.5 days of incubation), the growing feathers are detected to in the cornea.

When 5-day chick central corneal epithelium is associated with 7-day chick dorsal dermis for one month, in 2 cases among 3, the histology sections showed that an epidermis, stratum corneum involved in, formed from corneal epithelium under the influence of the dermis (**Fig. 28. A-B**). Likewise, in 2 of the 3 cases analyzed by immunofluorescence, Pax6 expression was downregulated in the nuclei of at least part of the corneal epithelium (**Fig. 28. C**) and K12 was no more expressed in the cytoplasm in the same area (**Fig. 28. D**). Similar results were obtained when 5-day chick central corneal epithelium was associated with 6-day quail dorsal dermis for one month. In 6 cases histologically analyzed there were 2 showed the formation of an epidermis. Likewise, immunofluorescence studies showed that Pax6 expression was downregualted and K12 no more expressed in 2 cases out of 3. Thus, in total, in 4 cases among 9, the 5-day corneal epithelium was transformed into an epidermis at least in some regions. However, feather morphogenesis was never observed, even at the feather bud stage.

In contrast, when the corneal epithelium originates from a 7-day chick embryo, associated with 7-day chick dorsal dermis, the 4 recovered cases showed no histological transformation of the corneal epithelium after one month (**Fig. 28. E-F**). In the 4 cases analyzed by immunofluorescence, Pax6 was expressed in the nuclei of the corneal epithelium (**Fig. 28. G**) and K12 was expressed in the cytoplasm (**Fig. 28. H**) as in the normal corneal

epithelium. The same results were obtained when the 7-day chick central corneal epithelium was associated with 6-day quail dorsal dermis (6 cases for histology analyses, 5 cases for immunofluorescence analyses). There was no stratum corneum formation and Pax6 and K12 were expressed as in normal corneal epithelium. Thus, the corneal epithelium kept its characteristics in 100% of cases (n=19), and the limited lability of the chick corneal epithelium at 5 days is no more present at 7 days of incubation.

IV. 2-4 Conclusion.

Chick corneal epithelium can be directly transformed into an epidermis under the guidance from an embryonic dermis, but only until 5 days of development. At an early stage, 3.5 days of incubation, it can even form feathers. At 5 days of incubation, in some parts of the recombinants, Pax6 decreased significantly in the nuclei, and at the same time, K12 was no more expressed in the cytoplasm of the same area. K12 expression is turned off in those cells where Pax6 is repartitioned from the nucleus to the cytoplasm and hence cannot act as a transcriptional activator. This result confirms that *keratin K12* is directly regulated by Pax6 (Liu, 1999). Two days later, the corneal epithelium keeps its characteristic even though it gets the signals from a dermis. Thus the chick corneal epithelium loses rapidly its ability to be transformed into an epidermis during embryonic development as previously suggested (Zak and Linsermayer, 1985). However, in contrast to what was published before (Zak and Linsermcyer, 1985) even at 5 days of incubation, the corneal epithelium is no more able to form feathers under the influence of a feather-forming dorsal dermis, and can only in part be transformed into an epidermis.

The avian corneal epithelium appears thus definitively committed at a very early stage. Around 4.5/5 days of incubation, the expression of *Pax6* gene become unable to be repressed by factors originating from the dermis. The corneal ectoderm at 3.5 days of incubation shows

a downregulation of Pax6 expression after one day of contact with a dorsal dermis and is directly transformed into an epidermis, and then involved in the formation of feathers. The non-formation of feathers by a 5-day, and a fortiori 7-day embryonic corneal epithelium might result from the non-formation of an epidermis (just in part at 5 days). In avian, all the thickness of the epidermis is involved in formation of feather buds in contrast to what happens in mammals where only the basal layer form the hair buds. Moreover the outgrowing of feather buds does not involve at the beginning dividing cells, but changes in cell morphology. We suggest that at an early embryological stage, the activation of *Pax6* is still labile in the chick corneal epithelium. Later, Pax6 expression, which might preclude cutaneous-appendage formation, appears to be autonomous in birds.

V. Can other epithelia be transdifferentiated into a corneal epithelium?

V. 1 Introduction.

In embryo and adult mammals, and even in birds at an early embryonic stage, a corneal epithelium has the ability to be transformed into an epidermis, forming hairs or feathers, under the guidance of an embryonic dermis. In reverse, whether or not can another epithelium be transdifferentiated into corneal epithelium by responding to an embryonic corneal or limbus stroma? Does this depend on a Pax6 expression by the epithelium?

V. 2 Materials and methods.

I used OF1, athymic mice, rabbits and eggs which come from the different companies or farms described as before (**Chapter I** and **IV**). Eggs were incubated at 38°C. The eggs were opened at 2.5 days according to the method described in **Chapter II**. Recombinants were grafted on nude mice kidney as described in **Chapter IV.1** (**Fig. 25**).

Recombinants were performed between different chick embryonic epithelia: 7 days dorsal epidermis, 9 days nasal epithelium, 8 days mouth epithelium, 7 days electroporated dorsal epidermis and different type of corneal stroma: 7 days chick embryo, 6 days quail embryo, 7 days postnatal mouse offspring. The cornea was recovered and dissected, cut in two pieces, which included both central cornea and limbus, or the cornea was dissected in two parts, central cornea and limbus. The corneal stroma was dissociated from the overlying epithelium after 2 hours treatment with saturated EDTA in Ca^{2+} and Mg^{2+} free medium. The chick nasal tissue was taken from the nasal cavity and the mouth tissue was taken from the cheek. The nasal and mouth epithelium were dissociated from the underlying mesenchyme by one-two hours treatment with the same medium as cornea. The epidermis and dermis from the

back of 7-day chick embryos were dissected and dermis and epidermis separated via protease treatment (1.25% trypsin + 2% pancreatine). The epithelia and corneal stroma were associated on agar medium for 2 hours and then grafted under the kidney capsule of nude mice using the method described as in **Chapter IV.1**. The grafts were recovered after operation between 2 to 4 weeks, embedded in OCT and stored at -80°C before cryosectioning for immunofluorescence by using Pax6 polyclonal antibody and K12 monoclonal antibody as described in **Chapter I**. To be sure about the quality of the recombinants, for the control I also used 11E10 monoclonal antibody (made in our laboratory), which is a general keratin antibody to check the epithelia.

For the electroporated chick epidermis, I opened the eggs as described in **Chapter II**. Then I injected mixed plasmid (*Pax6* and *GFP*) in the amniotic cavity, the left side of the embryo body. Two electrodes were put beside the embryo, the negative electrode in the left side, between the amnion and the dorsal ectoderm, the positive electrode in the right side, between the flank ectoderm and the amnion (**Fig. 29. A, B**). Then 20 volts and 10 sec lasting pulses were applied. In order to check the expression of GFP (**Fig. 29. C**), some of the embryos were recovered the day after electroporation, but most of them were returned to the incubator until 7 days of incubation.

The grafts were recovered between 2-4 weeks after operation.

V. 3 Results.

V. 3-1 Recombinants of chick embryonic dorsal epidermis and chick embryonic corneal stroma.

When 7 days dorsal epidermis is associated with different stroma, there are no difference between central corneal stroma and limbus stroma for the recombinants after 2-3 weeks

(**Table. 2**). In the group of recombinants between dorsal epidermis and central corneal stroma, among 4 cases, 4 (100%) formed a characteristic epidermis, with a statum corneum (**Fig. 30. A, D**). In 5 cases among 5 (100%), Pax6 and K12 were not expressed (**Fig. 30. G, H**). There were similar results when the dorsal epidermis was associated with the limbus stroma after 2-3 weeks: the histology (4 cases, 100%) as well as the non-expression of both Pax6 and K12 (7 cases, 100%) showed the formation of an epidermis.

V. 3-2 Recombinants of chick embryonic nasal epithelium and chick embryonic corneal stroma.

The results (**Table. 2**) are similar when 9 days nasal epithelium is associated with either 7 days central corneal stroma (the first group, 4 among 4 cases, 100%) or limbus stroma (the second group, 2 among 2 cases, 100%), demonstrating that the nasal epithelium keeps its characteristic as the formation of cilia of sensory cells (**Fig. 30. B, E**). Immunofluorescence staining (5 among 5 cases in the first group, 3 among 3 cases in the second group) shows that there are no Pax6 and K12 expression detectable.

V. 3-3 Recombinants of chick embryonic oral epithelium and chick embryonic corneal stroma.

When 8 days chick oral epithelium (**Table. 2**) is associated with chick corneal stroma (no matter its origin: central cornea, or limbus, or quail corneal stroma including both center and limbus), the oral epithelium formed a stratified squamous epithelium (**Fig. 30. C , F**), in total of 17 cases (see **Table 2** for details), and Pax6 and K12 expression are not detected (19 cases in total).

V. 3-4 Recombinants of chick electroporated dorsal epidermis and chick embryonic corneal stroma.

Even when the 7 days chick embryonic dorsal epidermis, which has been electroporated with Pax6 (**Table. 3**), is associated with corneal stroma including both center and limbus, there is formation of an epidermis with a stratum corneum (**Fig. 31. A, B**), 41 cases in total (see **Table 3** for details), and Pax6 and K12 expression are not detected (**Fig. 31. C, D**) in a total of 30 cases (see **Table 3** for details).

V. 4 Conclusion.

In chick embryo, the head epithelia (other than the corneal epithelium), or the dorsal epidermis, even after *Pax6-Pos* cDNA electroporation, are not able to transdifferentiate to corneal epithelium under the guidance of a corneal stroma. There are no difference between the induction of central corneal stroma and limbus stroma. All the epithelia keep their original characteristics. No Pax6 and K12 are detected in the epithelia of the recombinants.

Thus, an embryonic corneal stroma is insufficient to induce the expression of Pax6 which is one of the main corneal transcription factors, and K12 which is a specific corneal-type protein, resulting in a non transformation into a corneal epithelium. This is observed even with a nasal epithelium that express Pax6 at an earlier stage. By using *Pax6-pos cDNA* electroporated dorsal epidermis, we were unable to detect Pax6 expression. One explanation is that case Pax6 expression is labile and downregulated quickly, causing no enough Pax6 protein to allow an epidermis to transdifferentiate to a corneal epithelium. It should be better to use *RCAS-Pax6*-virus infection. The experiments are still in process. Recent works of Nakamura et al. (2003) and Nishida et al. (2004), successfully used the cultured mucosal tissue-engineered epithelial-cell sheets to transplant to the naked corneal stroma of human patients and rabbits. In rabbit, the overlying epithelium was removed or destroyed, and human

patients were diagnosed as having total limbal stem-cell deficiency. The important point is that they used cultured oral epithelium that might be obtained from stem cells. Here we used embryonic epithelia from the chick embryo, and at a developmental stage, the epithelial cells are still at an undifferentiated state and might contain numerous or be very close to stem cells. However, the transformation into a corneal epithelium was not obtained. Another point is that we used bird tissues, and the results might differ according to the species; our experiments with rabbit embryonic tissues are still in process.

The results form the Japanese groups might lead to the conclusion that oral adult epithelial stem cells can be induced to be transformed into a corneal epithelium by the corneal limbus. By using embryonic oral epithelial, we were unable to obtain a similar result.

CONCLUSION GENERALE ET PERSPECTIVE

Pax6 est exprimé au cours des premiers stades de l'organogenèse embryonnaire non seulement dans l'œil en formation mais aussi en particulier dans l'épithélium nasal et oral. La kératine K12, qui est directement régulée par Pax6 apparaît plus tardivement, lors de la différenciation de l'épithélium cornéen. Alors que Pax6 est rapidement réprimé dans les épithéliums nasals et œil, il continue à être exprimé tout au long de la vie adulte si que K12 dans l'épithélium cornéen. Au contraste de la kératine partenaire de K12 , K3, qui est exprimée aussi dans l'épithélium oral (Nakamura et al., 2003), K12 apparait être strictement spécifique d'une différenciation épithéliale de type cornéen.

Au contraire des résultats antérieurs obtenus chez la Drosophile (Gehring, 1996) et chez le Xénope (Chow et al., 1999), l'expression de Pax6 DP ne provoque pas la formation de structures oculaires ectopiques dans la tête. Nous confirmons ainsi les résultats publiés par un autre groupe (Kamachi et al., 2004) au cour de nos propres travaux. Nos résultats montrent de plus la nécessité d'une régulation fine de la quantité et la localisation de Pax6, car une surexpression détruit l'orientation dorso-ventrale de l'œil.

Nous avons montré que le cristallin n'est pas requis, ni pour la migration des cellules mésenchymateuses formant le stroma, ni pour l'expression de la kératine 12 dans l'épithélium cornéen, mais uniquement pour la croissance du globe oculaire dans son ensemble. Nos résultats suggèrent que l'épithélium cornéen est spécifié très précocement, soit si multaniment à l'incubation de la placode cristallinienne, soit juste après, par la vésicule optique, ou par le bord de celle-ci, qui formera l'iris par la suite. L'épithélium cornéen se différencie ensuite en permettant la migration des cellules mésenchymateuses. Ceci est en accord avec des résultats non encore publiés d'une équipe du laboratoire de P. Chambon. En effet les expériences de N. Matt viennent de montrer que la synthèse de raldh par l'ectoderme de la future cornée permet

à partir du rétinaldehyde la formation d'acide rétinoique, ce dernier étant requis pour la formation fu stroma.

Donc dans une première étape, la vésicule optique ou le futur iris, induirait l'individualisation du futur épithélium cornéen. Dans une deuxième étape l'épithélium cornéen permettrait la formation du stroma. Le dernier joue-t-il un rôle dans la différenciation de l'épithélium cornéen? Il était logique de le supposer vu le rôle prépondérant du derme dans la différenciation de l'épiderme (Dhouailly, 1977 ; Olivéra-Martinez et al., 2004). Le stroma pourrait être requis au tout début de l'organogenèse de la cornée (troisième étape) puis l'épithélium cornéen deviendrait totalement autonome. En effet, l'insertion d'un fragment de derme sous l'ectoderme de la future cornée à 3.5 jours d'incubation chez le poulet produit la répression de l'activité de Pax6 et al transformation de cet ectoderme en épiderme producteur de plumes. Cette transformation ne peut plus être obtenue que partiellement à 5 jours et à 6 jours l'épithélium cornéen de poulet apparaît définitivement déterminé.

Au contraire, l'épithélium cornéen de lapin même adulte est capable sous l'influence d'un derme embryonnaire de souris de donner naissance à des follicules pileux, puis un épiderme. Dans un premier temps, l'expression de Pax6 puis de K12 sont réprimées.

Ces résultats non similaires sont-ils dus à des capacité différentes de l'épithélium de cornée, ou du derme embryonnaire, chez les oiseaux et les mammifères? Les nouvelles expériences entreprises dans le laboratoire permettront de répondre à cette question.

La transformation inverse, d'épithélium divers, par exemple l'épithélium oral en épithélium cornéen est un enjeu très important du point de vue médical. Mes expériences de recombinaisons de stroma cornéen ou limbal et de divers épithéliums ont donné des résultats négatifs et remettent donc en question ceux publiés durant la dernière partie de préparation de ma thèse par deux groupes (Nakamura et al., 2003 ; Huang et al. 2004).

De nombreuses autres questions restent en suspens et ouvrent de nombreuses perspectives : l'épithélium cornéen est spécifié très tôt au cours du développement, et le stroma ne jouerait qu'un rôle secondaire. Quelle est la nature moléculaire du message isu de la vésicule optique? Pourquoi l'expression de Pax6 continue-t-elle tout au long de la vie dans la cornée et est-elle éteinte très rapidement dans l'épithélium nasal ou oral? Le stroma cornéen ou limbal joue-t-il un rôle dans ce maintien? Pourquoi l'expression de K12 apparaît tardivement? Quel co-facteur pourrait être requis, bien que Pax6 régule directement le promoteur de K12?

Notre première hypothèse de travail était que des épithéliums exprimant Pax6 tel que l'épithélium oral ou nasal pourraient être transformés en épithélium cornéen une fois associés à un stroma cornéen hors il n'en est rien. Il convient de notre qu'au moment de l'association ces épithéliums n'expriment plus Pax6.

J'ai essayé de sur exprimer Pax6 en réalisant des électroporation avec un plasmide Pax6 DP. Dans la région de la tête, cette surexpression n'est pas suffisante pour déclencher la formation de structures oculaires ectopiques, mais aboutit à une désorganisation de l'axe dorso-ventral de l'œil. J'ai essayé également de sur exprimer Pax6 par électroporation du même plasmide dans l'épiderme embryonnaire, en électroporation à 3.5 jours d'incubation et en prélevant l'épiderme à 7 jours d'incubation pour réaliser des recombinaisons. Les résultats ont été négatifs. L'expression de Pax6 obtenue ainsi était sans doute transitoire.

De nombreuses questions restent donc à résoudre avant de pouvoir obtenir un épithélium cornéen à partir d'autres cellules dans un but thérapeutique.

GENERAL CONCLUSION AND PERSPECTIVES.

In early chick embryo, *Pax6* is expressed in the nuclei not only in the developing eye tissues, but also in other head structures and particularly in nasal and oral epithelia. After corneal epithelium individualized, *Pax6* expression continues in this tissue through all the embryonic stage and the life of the adult, whereas it is downregulated until no more expressed after 7 days of incubation in the other head epithelia of ectodermal origin. In mammals, we were not able to check for *Pax6* expression in rabbit. In human, the Pax6 protein is present in both nuclei and cytoplasm of the adult central corneal epithelium and in the cytoplasm of the basal layer of the limbus. *K12* expression is strongly detectable in the cytoplasm of all the layers of the corneal epithelium from a late embryonic stage, 14 days in chick, 21 days in rabbit, and throughout all the life of the adult. In human, we confirm that *K12* is expressed in the cytoplasm of the adult corneal epithelium, but we were unable to analyse foetal situation. However, no matter of the species, avian or mammals, there are no *K12* expression detected in the nasal or oral epithelia, and thus this keratin is clearly specific of corneal epithelium differentiation, in contrast to its partner, *K3*, which is also expressed in oral epithelium (Nakamura et al., 2003).

In contrast to what happens in Drosophila and Xenopus, there is no supplementary eye structures formed after transfection with *Pax6-Pos*, even in the head ectoderm. We thus confirm the results obtained by another laboratory (Kamachi et al., 2001; 2004) during the course of this thesis. We propose that for corneal epithelium commitment and maintenance, a complex of Pax6 and another transcription factor resulting from a signal from the iris, then limbal mesenchyme might be required. Moreover, although it is known that *K12* is controled by *Pax6* (Liu et al., 1999), the question remains to know how and why it is triggered only by a late stage. On another hand, our experiments show that whereas the electroporation of *Pax6-*

Neg impeded the formation of eye structures, the electroporation of *Pax6-Pos* lead to disruption of the normal dorsal-ventral patterning of the eye. The choroid fissure, which is normally localized in the ventral region of the retina was mislocalized to the dorsal region, showing the importance of the amount and distribution of *Pax6* transcripts in the dorso-ventral orientation of the eye.

We show that the lens which is a source of FGF10 (Govindarajan et al., 2000) is only required for the general and harmonious growth of the eye, but not for the migration of fibroblasts of the corneal stroma nor for the differentiation of the corneal epithelium and K12 expression. Our results suggest that the optic cup, not the lens, has the first inductive effect on the still undifferentiated ectoderm. The contact between the optic neural vesicle and the ectoderm might induce concomitantly both the lens and the corneal epithelium, or alternatively, the corneal ectoderm might be specified soon after by the future iris. Then the corneal epithelium interacts with the mesenchymal neural crest cells and allow them to migrate and form the corneal stroma.

When an adult rabbit corneal epithelium is associated with an embryonic mouse dorsal dermis, *K12* and *Pax6* expression are downregulated in the basal layer of the corneal epithelium. It should be noted that in mammals, the downregulation of Pax6 gene activity starts rapidly in the basal cells of the adult corneal epithelium, and the Pax6 protein is no more detectable in the growing hair buds. In this case, the formation of an epidermis originating from an adult corneal epithelial cells is undirect: it occurs secondarily, from hair stem cells which appear after the formation of complete hair follicles (Ferraris et al., 2000; Pearton et al., 2004). It would be interesting to know whether in the recombinants involving a corneal epithelium from a 12-day rabbit embryo (Ferraris et al., 1994), which starts rapidly to form hair buds, the transformation into an epidermis might be a direct event. Indeed, we show here that at a corresponding stage (3.5 days), Pax6 is rapidly downregulated in the ectoderm

and the embryonic chick corneal epithelium is directly transformed into an epidermis under the guidance from an embryonic dermis. However, in contrast to mammals, the avian corneal epithelium appears definitively committed, during the 6th day of incubation: Pax6 can not be even downregulated in the basal layer cells of the recombinants. We suggest that at an early embryological stage, the activation of *Pax6* is still labile in the corneal epithelium in vertebrates. Later, *Pax6* expression, which might preclude cutaneous-appendage formation, appears to become autonomous in birds, more precisely after the formation of the corneal stroma (5 days of incubation), but to remain still labile in mammals even in the adult. Another interpretation is that *Pax6* might only completely be downregulated in highly dividing cells of heterotopic recombinants like those of the forming hair peg. Moreover, we confirm that *K12* is directly regulated by *Pax6* in both species.

Reversely, the head epithelia other than corneal as well as the dorsal epidermis in chick embryo are not able to transdifferentiate to corneal epithelium under the guidance of a corneal stroma. No *Pax6* and *K12* expression are detected in the epithelia of the recombinants. Even an embryonic corneal stroma or limbal stroma is insufficient to induce the expression of *Pax6* into embryonic head epithelia, which have expressed Pax6 at an earlier stage (Tiffany et al., 2002). Comparing the results from a Japanese group (Nakamura et al., 2003) and a Chinese group (Huang et al. 2004) who successfully used respectively cultured mucosal epithelial-cell sheets or epidermal stem cells to transplant to the naked corneal stroma of human patients and rabbits, the transformation to a corneal epithelium might depend on the species, or on how much stem cells are invoved in different experiments. An alternative is that in their experiments the corneal epithelium is regenerated from a few remaining corneal cells of the host. The recombinants that we did in mammals between the head epithelial other than the cornea and corneal stroma are currently in analysing process and will be presented at the oral.

These different results raise several questions that will need future molecular biological coupled with microsurgery researchs. *Pax6* appears to be autoregulated in the chick corneal epithelium after the formation of the corneal stroma. How its expression is inhibited very rapidly after the contact of a 3.5-day chick embryo corneal epithelium with a dermis? How this rapid inhibition can occur in rabbit even in the case of an adult corneal epithelium? Why the Pax6 protein is only found in the cytoplasm of limbal basal cells? Why *Pax6* continues to be expressed in the adult corneal epithelium whereas it is completely downregulated during the second part of embryonic development in nasal and oral epithelia? Our experiments with *Pax6-Pos* electroporation of the epidermis were not conclusive because *Pax6* is downregulated rapidly after electroporation. Whether using *RCAS-Pax6* infection will lead to different results will be interesting to know. In the case of a stable *Pax6* expression in epidermal cells, does the contact with an embryonic corneal stroma would be sufficient to obtain its transdifferentiation into a corneal epithelium? However, we suspect that not only *Pax6* expression is not sufficient but that corneal specification requires two groups of other factors which can be upregulated following first the ectodermal contact with the future iris, and later by the time of K12 expression, with corneal or limbal stroma. Finally how to explain the contradiction between our results and the formation of a corneal epithelium from oral cells (Nakamura et al., 2003)? I think that it is only when these different questions will find an answer that corneal cell therapy could be routinely successful.

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