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Universidad Cumplutense
28040 Madrid. Spain

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Lectures

THE INITIAL STAGES IN OTOCYST DEVELOPMENT

E. Barbosa Ayúcar

Dept. of Morphological Sciences, University of Valladolid

Our work focuses on the initial phases of the inner ear, whose morphological characteristics are similar to those of other sensory organs (eyes and nose). The successive phases are calles PLACODE, FOVEA, VESICLE AND OTOCYST.

Devolopment of the inner ear begins with the formation of the OTIC PLACODE, a thickening of the cephalic ectoderm at rhombencephalic level. At the level of the placode, flattened polygonal ectodermal cells are transformed into cubic-cylindrical cells having apex-base polirity; the rounded apical surface has scanty microvilli and the basal pole "rests" on the basalmembrane of this incipient epithelium.

Continuity in these initial phases of the neuroectoderm of the neural folds with the ectoderm that forms the otic placode has enabled description of the neuroectodermal origin of the medial portion of the placodeal epithelium, in which the cells of the neural ridge would be included.

The otic placode grows progressively and initiates an asymmetric sinking, acquiring the FOVEA conformation, with its medial portion –whose epithelial elements have a basal membrane– appended to the neural tube, of which at least laminin, collagen IV and chondroitin sulfate proteoglycan are components. By contrast, the epithelial cells forming the lateral portion of the fovea or floor lack a basal membrane.

During this stage of development, the placode epithelium is cytologically homogeneous, with the exception of the structural difference (presence/absence) as regards the basal membrane; the rounded surface of the foveal cells has numerous microvilli and a single cilium.

Recently, reports have been made of the asymmetric expression of genes (encoding transcription factors, secreted proteins or receptors) whose meaning requires clarification and further study but which could be related to the establishment of the axes of the anlage with respect to the embryonic globals, thus establishing a division of the same into compartments; it also seems to be apparent that the vestibular structures are programmed before and in a different way from the cochlear portion.

This diversity in gene expression at this stage of the fovea is not unique since antibodies such as HNK-1 reveal the existence of two cellular populations, one expressing the epitope recognizable by the antibody (3-sulfoglucuronic acid) and the other not expressing it. Previously, descriptions have been made of the presence of certain enzymes (acid and alkaline phosphatase) circumscribed to certain portions of the anlage.

The data could be summarized thus: gene differentiation occurs before morphological differentiation and may determine the final fate of each part of the anlage. The auditory fovea, whose walls initially form a dihedral angle of ±90°, progressively closes its angle through an "elevation" of its horizontal flat wall which becomes vertically oriented and "seeks out" the free edge of its internal or neural wall which is soldered to the lateral surface of the neural tube with which it shares the basal membrane.

The approximation of the "edges" of the fovea eventually fuses them and a varying number of apoptotic cells appears at this level. The role of these cells in the fusion and later isolation of the vesicle of the ectoderm is still subject to debate.

At the same time, the vesicle/otocyst separates from the neural tube and cells of the mesenchyma become intercalated between both anlage.

Acoordingly, the isolation/independence of the ectoderm and separation of the inner wall of the visicle/otocyst of the neuraltube are the morphological characteristics defining this stage of development.

Away from the ectoderm, the endolymphatic duct is rapidly conformed in the otocyst; its epithelial elements are clearly differentiated from the rest. As from this phase of development, the otic anlage of birds can configure its eight sensory receptors with an adequate structure for their function.

Likewise, later reports have been made of the expression of BMP-4 (Bone Morphogenetic Pro-

tein), MSX-1 and P75 NGFR in the cristae, lagena and macula neglecta.

The epithelium, be it ectodermal, neuroectodermal or of both types, from which the otic placode is developed does not only originate the fovea, vesicles and otocyst, but also neuroblasts, from which the adult ganglia are eventually formed. The glial elements of these ganglia could derive from the neural ridge of R3-35 rhombomeres trapped in the neuroectoderm, as suggested by Mayordomo et al. (1998). This hypothesis seems to support the notion of a population reactive to HNK-1 in the foveal phase as well as its migration to the acoustic-facial ganglion as detected by DIL/DIO labelling.

MOUSE KIDNEY DEVELOPMENT: SOLVED PROBLEMS AND UNANSWERED QUESTIONS

Jonathan B.L. Bard

Department of Biomedical Sciences, Edinburgh University, Edinburgh EH8 9AG

The mouse metanephric kidney starts to form at about E11 when the ureteric bud, a diverticulum off each caudally extending nephric duct, extends medially towards the nearby metanephric blastema, a small condensation of metanephric mesenchyme (MM) located within each intermediate mesoderm, that induces it to arborise and make what will become the collecting-duct system. Soon after it contacts the blastema, the bud induces small groups of nearby mesenchymal cells to condense, epithelialise and differentiate into nephrons that fuse to the growing duct system. Over a period of a week or so, a population of stem cells that become localised at the periphery of the growing kidney continue to produce about 1500 such aggregates so that, for much of kidney development, there is a graded progression in nephron differentiation, from just-formed ones at the periphery to more mature ones at the cortico-medullary border.

The talk will briefly review these events and the experimental tools for investigating the mechanisms uderpinning them, and then try to answer some key questions about kidney development as there has been considerable progress in the field over the past few years. Work from several laboratories, using a mix of classical embryological and contemporary molecular techniques, has shown:

- How expression of the transcription factor WT1 has enabled the MM competence to induce the formation and invasion of the ureteric bud through the expression of the signalling factor GDNF.
- That the MM responds to the inductive signal from the ureteric bud by the expression of a series of genes that includes transcriptions factors, and signalling and adhesion molecules.
- The nature of the reciprocal inductive interactions between the MM and the ureteric bud, although some of the molecular details remain to be elucidated.

Much about kidney development is, however, still unknown and the talk will also consider some of these the unsolved problems. Here, a distinction is made between those problems where we can, in principle, formulate a solution, although finding it may be technically "difficult", and those where we still lack a conceptual framework withing which to search for molecular data and are thus "mysterious". Among the former are the linking of nephrons to the collecting-duct arborisation; the responses to the expression of transcription factors, the regulation of stem cells, the identification of the various cell types in the early metanephric blastema., and the formation of the vascular system and its linking to the glomerulus. Among the latter are the formaton and differentiation of nephrogenic aggregates, their differentiation with its 1-D pattern formation and the formation of the collecting duct tree.

THE GENETIC CONTROL OF EYE DEVELOPMENT

D.R. Davidson

Despite rapidly accumulating evidence from gene expression assays, knock-outs and ectopic expression experiments, many of the most important questions concerning the genetic control of eye development remain to be solved. One particularly interesting question that has received a lot of attention recently is what regulatory mechanism distinguish the different regions of the optic cup?

Pointers to the genetic interactions that determine the boundary between optic stalk and retinal pigmented epithelium (RPE) have come from recent work on the Sonic Hedgehog signalling pathway and it's action on several genes including Pax2. The mechanism that ditinguish developing neural retina (NR) from RPE are less clear. In common with many of the determinative processes in early eye development, these patterning decisions appear to involve signalling by Fibroblast Growth Factors, but the subsequent effects on transcription factors regulating programmes of gene activity are not understood.

One family of transcription factors that is likely to be involved in this process is the *Mxs* genes, related to the *Drosophila* gene *msh* and one of the most highly conserved families of homeobox-containing genes. In the mouse, *Msx1*, *Msx2* and *Msx3* are expressed in overlapping domains in the dorsal neural tube (Davidson, 1995; Shimeld et al., 1996) while *Msx1* and *Msx2* are expressed in the prospective neural retina, but not in the prospective RPE. *Msx2* is expressed in the prospective neural region of the early optic cup, while *Msx1* is expressed later, in the prospective ciliary region at the boundary between neural and non-neural (pigmented) tissue (Monaghan et al., 1991). Comparative evidence from *Drosophila* suggests that these patterns may reflect a true involvement in the process of patterning and neurogenesis. In particular,

loss -of-function mulations in *msh* lead to changes in the fate of neuroblast in the dorsal neurectoderm indicating a role in neurogenesis, while ectopic expression throughout the neurectoderm severely disrupts the development of midline and ventral structures (Isshiki et al., 1997), suggesting a role in normal dorso-ventral patterning. Genetic evidence supports the idea that the *Msx* genes also play a role in the development of the neuro-epithelium in the vertebrates, particularly with reference to the eye. Though an eye phenotype has not been detected in loss-of-function mutatons in either *Msx1* or *Msx2* (Satokata and Maas, 1994; Rauchman, 1997), the double mutant $Msx1^{-/-}$ $Msx2^{-/-}$ showed phenotypes varying from arrested development at the optic vesicle stage to general microphalmia, indicating that while the genes have redundant functions they are nevertheless requiered for normal eye development. This evidence is supported by anti-sence RNA experiments aimed at disrupting *Msx1* and *Msx2* function which caused eye defects including enlarged optic vesicles and micropthalmia (Foerst Potts and Sadler, 1997). These experiments indicate, in a general way, that Msx1 and Msx2 are important for eye development, but they do not address the question of what is the role of the genes in relation to their specific sites of expression.

I will describe some of our recent experiments aimed at beginning to address this question. In this work we have extended our original studies of *Msx* expression in the mouse eye and further investigated the functions of the genes by ectopic expression *in vitro*. We confirm that *Msx2* is expressed in the prospective neural region of the optic vesicle at E9, but find that, like *Msx1*, *Msx2* is expressed in the prospective ciliary region at the boundary between neural and non-neural epithelium. While *Msx1* is expressed around the circumference of the eye cup, however, the expression of *Msx2* is confined to the dorsal region. These expression patterns suggest that the genes might play a role in patterning neural/non-neural differentiation of the optic vesicle and developing optic cup. To examine this possibility, we expressed *Msx2* in chick RPE cells in culture. We find that *Msx2* expression results in the development of a neural morphology phenotype in a small number of cells and a more widespread increase in the expression of the neuronal marker, Class III β tubulin. In addition, we find that *Msx2* expression leads to a marked decrease in the numbers of cells positive for Mitf. These results suggest that one function of the *Msx* genes may be in the process that partitions neural and non-neural tissues in the optic cup.

FIRST PHASES IN EYE DEVELOPMENT: MORPHOGENESIS OF THE LENS

J. García-Porrero

University of Cantabria

The first morphological manifestation of the embryonic eye is an evagination in the lateral walls of the prosencephalic vesicle, called the optic vesicle, a neuroectodermal tissue that later forms the retina. Through an interphase, the optic vesicle apposes to a specific territory of the lateral cephalic ectoderm. Against the optic vesicle the ectoderm undergoes considerable alterations that eventually convert it into the lens.

Here we report the early development of the optic vesicle; in particular lens morphogenesis. We offer a critical review of the cytological and histological aspects that determine the formation of the lens placode, its invagination in the fossa of the lens, the process of shedding of the anlage of the rest of the cephalic ectoderm and the evolution of the lens vesicle to be differentiated into a lens transparent to light.

Based on experimental work, we analyze the role in these morphogenetic processes played by microtubules, microfilaments, the cell cycle rhythm, programmed cell death, extracellular material and adjacent tissues. Finally, it is suggested that differences in the composition of the lens capsule may contribute to delimiting the proliferative and differentiation compartments of the lens.

HISTOGENESIS OF THE INNER EAR

Pablo Gil-Loyzaga

Centro de Cultivos Celulares (CAI) and Depto. Cirugía II. Fac. Medicina, Universidad Complutense de Madrid

The maturation and final differentiation of the epithelium of the auditory receptor starts just at the end of the spiral growing of the cochlea, and follows a base-apex progressive gradient. The base of the scala media could be now divided into two epithelial ridges: the inner, from which derive the spiral limbus, Kölliker's organ and the major part of the tectorial membrane (TM), and the outer, anlage of the organ of Corti and a minor part of the TM. The development of the TM results in the progressive disappearance of the Kölliker's organ and the subsequent opening of the inner spiral sulcus. The development of the outer spiral sulcus results in the differentiation of sensory and supporting cells of the organ of Corti. This part of maturation is accompanied by the auditory receptor innervation. This presentation focuses in the differentiation of both inner and outer epithelial ridges.

The inner ridge suffers, first, some phenomena of growing, then the secretion and maturation of the TM and, lastly, a progressive involvement and degeneration process including apoptosis. After a general overview of these processes, a special commentary will be devoted to the TM maturation. The TM is an acellular structure, strongly hydrated (near to 90% containing water), biochemically composed by glycoconjugates (glycosaminoglycans among others), collagen and non-collagenous proteins. TM is a very important structure involved in the mechanical deflection of sensory cells stereocilia, but also as a probable selective ion exchanger between endolymph and the apical surface of the organ of Corti.

From the outer epithelial ridge mainly derive the sensory cells: inner and outer hair cells. Their maturation is accompanied by a significant epithelial reorganization, being the opening of Corti's tunnel and Nuel's spaces, and the arrangement of the reticular lamina some primary events. Also, the concomitant appearance of both afferent and efferent nerve fibers in still immature epithelium is conspicuous. First fibers reaching the epithelium (recognized as afferent elements) could be recognized largely before the differentiation of the sensory cells, by using electron microscopy, silver staining techniques, and immunocytochemistry (GAP-43). Experiments carried out in an in vitro model supports that nerve fibers are a primary element in the hair cell differentiation. These nerve fibers are sensitive to glutamate (the main afferent neurotransmitter of this sensory receptor in the adulthood), and its agonists, from a very early period of the spiral ganglion neuron development. Several experimental model carried in vivo and in vitro strongly suggest that glutamate could serve as a neurotrophic factor during cochlear development.

Progressively, the organ of Corti's epithelium is reached by efferent fibers originated in the olivocochlear nuclei complex. These fibers early express several neuroactive substance and neurotransmitters in a subsequent chain of morphological events. Sequential studies carried out in the rat and guinea pig cochlear maturation, by using immunocytochemical methods, have provide a clear schedule of the timing of appearance of synaptophysin (a major synaptic molecule), acetyl choline and CGRP, GABA and GAD, enkephalins and catecholamins. Neuronal plasticity suggest an important remodeling of nerve fibers that started to synthesize neurotransmitters.

LIMB MORPHOGENESIS: CONTROL OF CHONDROGENESIS AND OF CELL DEATH

Juan M. Hurlé

Department of Anatomy and Cell Biology, University of Cantabria

Limb outgrowth occurs distally as the result of the effect of the apical ectodermal ridge (AER) on the underlying mesenchyma. This zone of the mesenchyma is known as the zone of progress. During development, cells of the zone of progress undergo a process of differentiation into cartilage or are eliminatee by apoptotic cell death. In the present work, we review the signals responsible for this dual fate of cells from the zone of progress.

Our data indicate that apoptotic fate is mediated by growth factors from the family of Bone Morphogenetic Proteins (BMP) while chondrogenic fate depends on the action of members of the TGFβ family and on activins. Additionally, we show that these factors establish a complex interaction in such a way that BMPs also induce cartilage growth when TGFβs and activins condition cells by the induction of a BMP type-1 receptor.

Finally, we analyze the role of antagonists of BMPs as modulators of cartilage growth and the role of activin antagonists in tendon differentiation.

GENETIC CONTROL OF DIGITAL NUMBER IN MAMMALIAN LIMB DEVELOPMENT

G.M. Morriss-Kay, C. Hayes* and S. Iseki

*Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, U.K. and *MRC Mouse Genome Centre and Mammalian Genetics Unit, Harwell, Didcot, Oxon, UK*

Formation of digits in the vertebrate limb is under the genetic control of interacting signalling systems. Fibroblast growth factors (FGFs) play key roles in stimulating cell proliferation during early limb bud formation and outh growth, in expanding the digit-forming mesenchyme of the limb autopod (han or foot plate), and in maintaining the alternating pattern of skeletal and connective tissue that is essential for morphogenesis of the digits as separate skeletal elements.

The basic number of digits in mammalian limbs is five, although this has been reduced in some groups. Differences in length of digits 1-5 give the hand plate anteroposterior polarity, e.g. the most anterior mammalian digit, the pollex or hallux, has only two phalanges. Digital number and polarity is controlled by signals emanating from the zone of polarising activity (ZPA), a region of posterior limb bud mesenchyme in which the gene *Sonic hedgehog (SHH)* is expressed. SHH signalling induces expression of *FGF4* in the adjacent (posterior) AER. Interaction between the SHH and FGF4 signalling systems produces a region of rapidly proliferating digit-generating mesenchyme in the posterior half of the expanding autopod. Digital number depends on tghe amount of mesenchyme generated, while anteroposterior digital polarity is thought to depend on the strength of a signal diffusing from the ZPA.

Following the discovery of *SHH*, it was thought that diffusion of SHH protein from the ZPA was the basis of digital polarity. However, it is now known that SHH is unable to diffuse away from its site of secretion, suggesting that its role may be to activate a second secreted factor. A new polydactylous mouse mutant, *Doublefoot (Dbf)*, has revealed a possible candidate molecule for this function. *DBF* is a dominant, activating mutation. The polydactylous phenotype does not show anteroposterior polarity. All parts of the distal limb bud mesenchyme are able to induce extra digits when grafted to the anterior side of the chick wing bud, i.e. the whole of the distalmesenchyme acts as one large ZPA. Surprisingly, *Shh* expression is confined to its normal posterior domain; in contgrast, *Fgf4* is expressed throughout the AER, and the whole distal mesenchyme shows a high level of cell proliferation. These observations suggest that the normal DBF protein is a secreted molecule that requires activation by SHH in the ZPA, from where the acti-

vated molecules diffuse anteriorly, inducing signalling via the SHH pathway in the digit-forming mesenchyme in the posterior half of the developing autopod. In contrast, the mutant Dbf protein is constitutively active, inducing *Fgf4* throughout the AER and activating the Shh pathway throughout the autopodial mesenchyme, which expands to generate a greatly increased number of digits.

These experiments indicate that the primary role of the ZPA is to stimulate mesenchymal proliferation in the developing limb. The number of digits formed is dependent on the amount of mesenchyme generated; this is regulated by a mesenchymal-epithelial tissue interaction whereby the Dbf protein modulates Fgf4 expression in the AER.

The proper regulation of FGF signalling is also vital for morphogenesis of the digits as separate skeletal elements. Apert syndrome is a human abnormality caused by mutation of the FGF receptor FGFR2. FGFR2 has two isoforms, which are activated by different FGF ligands. *FGFR2-IIIb* is expressed in the surface ectoderm and around the digital cartilages, and *FGFR2-IIIc* is in the interdigital mesenchyme. The Apert phenotype includes severe bony syndactyly of the hands and feet. The mutation causes ectopic expression of the *FGFR2-IIIb* isoform in the interdigital mesenchyme, which is the likely cause of fusion of the digital bones. The differential digital/interdigital and epithelial/mesenchymal expression patterns of the two FGFR2 isoforms generates an alternating pattern of FGF signalling that is clearly essential for maintaining digital separation.

MORPHOLOGICAL STUDY OF AN EXPERIMENTAL MODEL OF RENAL POLYCYSTOSIS INDUCED BY CORTICOSTEROIDS

José L. Ojeda

Department of Anatomy and Cell Biology, University of Cantabria (Spain)

Renal polycystosis (RP) is a disease in which some or all of the nephrons and/or collecting ducts become dilated. Essentially, the cysts are giant segments of nephrons or collecting ducts that have preserved their function. Accordingly, in RP the mechanisms responsible for regulating the size of the different segments of the nephrons or collecting ducts must be altered.

Size is an essential part of organic forms that is often not taken into account. Study of PR could shed light on the pathogenesis of the disease and on the mechanism controlling normal kidney development.

Here we studied an experimental model of PR induced by the injection of methylprednisolone acetate at a single dose of 20 mg/kg b.w. to newborn rabbits. This model induces cyst formation in 100% of the cases, and the cysts can be divided into two types: a) dilations of the collecting ducts, which revert with time and b) glomerular cysts due to dilation of the urinary space of the renal corpuscle. Both types communicate with the other parts of the urinary system.

The basic information provided by the cysts due to dilation of the collecting ducts is as follows: First, the epithelium of the cyst has a normal structure and, qualitatively and quantitatively, both cell populations that normally form the collecting ducts are conserved: these are the main cells and the intercalated cells, with their four sybtypes. This confirms that cysts of the collecting ducts are in fact giant collecting ducts that conserve their normal structure. The most striking observation is the alterations to the basal membranes (BM), which appear early on and are specific to the collecting ducts that are in the process of developing cysts. Morphologically, the main alterations consist of a swelling and multilayering of the lamina densa. Using Ruthenium Red, an increase in the number of anionic points of the altered membrane is seen. Immunocytochemical studies reveal large increases in fibronectin and tenascin levels associated with the altered BM. The increase in fibronectin is consistent with the observation that glucocorticoids stimulate the synthesis of this glycoprotein. Tenascin is expressed transiently during renal development and is never found in the BM. Tenascin is an oncoprotein that is intensely expressed in some tumours. The abnormal presence of tenascin in the BM seems to support the tumoral theory of RP.

The precociousness of the alterations to the BM, the selectivity of such alterations and their disappearance when the cyst regress all suggest a direct relationship between them and cyst formation in the collecting duct. The question then arises as to how the altered BM is able to induce cystic dilation of the collecting ducts. The BM is thickened such that its compliance mode, and hence its elasticity, are decreased. Also, the cysts intercommunicate with one another and hence a mechanical hypothesis to account for cyst formation through simple distension should be ruled out. The extracellular materials (EM) provide signals through integrins that control many cellular functions. Alterations in the BM may modify the signals controlling normal growth of the collecting ducts and lead to their cystic dilation. Among other factors, the normal size of the collecting ducts must depend on EM.

The most significant observation in glomerular cyst (GC) is that the parietal leaf is occupied by podocytes. Parietal podocytes (PPs) have the same phenotype as visceralpodocytes. Their developmental sequence indicates that they are produced by metaplasia. PPs develop without the presence of capillaries. These cells represent a unique model for studying the biology of podocytes, but the question remains as to which factors control podocyte differentiation. Their differentiation in the absence of capillaries indicates that no endothelial factors are required.

PPs permit direct analysis of the functional capacity of podocytes *in vivo*. The use of electgron-opaque tracers (ferritin and cationic iron, and

peroxidase) of different sizes shows that the passage of molecules through the filtration clefts is not polarized.

Normally, the glomerular BM (GBM) is formed through the fusion of two basal membranes, one coming from the endothelium and the other coming from podocytes, and has its own ultrastructural and immunohistochemical characteristics that differentiate it from other renal BMs. The main issue is therefore which of these characteristics derives from the endothelium and which derive from podocytes. Lacking an endothelium, PPs are a unique model for addressing this issue. The inner lamina rarae only appears when the BM of PPs contacts interstitial cells; if not, it is missing or a lamina fibroreticularis takes his place. After treatment with guanidine, the BM of PPs only displays an electron-dense layer, the electron-lucent one being absent. This shows the different origins of the components dissociated by guanidine. After the use of different oxidants (periodic acid or permanganate) and enzymatic digestions (elastase or lysozyme), the silver method of Jones reveals the different affinities for silver of the BM of the normal renal corpuscle. This method shows that the BM of PPs does not show the typical silver affinity of the GMB, owing to the absence of the endothelial component.

The different BMs of the normal renal corpuscle show a characteristic lectin staining patter. The GMB stains intensely with WGA, PNA (after neuraminidase) and MPA, whereas the parietal BM does not. In GC, the BM of PPs displays the same labelling pattern as the GMB, indicating that the special glycosylation pattern of this BM is due to its podocyte component.

DEVELOPMENT OF THE VERTEBRATE RETINA

F. Prada Elena

Department of Morphological Sciences, University of Seville

It is not easy to discuss the development of the retina if one takes into account that this structure represents one of the most important sites for study of the nervous system. There, we take as reference the chick retina since, if not the best, it is ceertainly one of the better models for studyng the development of the vertebrate retina. Only from the point of view of genetics does the mouse retina surpass that of the chick since many spontaneous mutations have occurred in this species and mouse genetics are well known.

As in other nerve structures, in the retina the developmental program occurs in succesive stages, discussed below and based on previous work by this team.

1. The cellular proliferation stage allows the neuroepithelium of the retina to generate a specific number of cell divisions, which is different for each neuronal population. Stem cells finish their proliferation and after the last division leave the cell cycle transformed into immature neurons whose fate is to survive throughout the life of the animal. The date of birth is understood to be the moment corresponding to the last cell division and this may differ or coincide in some types of neurons.

From autoradiographic studies, we now know that in the retinas of non-mammals the order of cell generation is as follows: (1) ganglion cells, (2) horizontal, amacrine and photoreceptor cells, (3) Müller cells, (4) bipolar cells. In the case of photoreceptors, the cones always appear before the rods (Fujita and Hori, 1963; Kahn, 1974; Spence and Robson, 1989 and Prada et al., 1991). In the mammalian retina the order of appearance is similar: (1) ganglion cells, (2) amacrine and horizontal cells and cones, (3) bipolar and some amacrine cells (4) Müller cells and rods (Harman et al., 1992).

In this section on development, the contribution of Dr. Carmen Prada's team has been decisive because it has analyzed cytogenesis in greater depth and has demonstrated that the process of cell generation in the retina occurs following gradients ranging from the dorso-central to the ventro-central regions, first passing through the temporal retina and then the nasal retina. The peripheral retina develops later on, following the same gradients.

Currently, much effort is being devoted to gaining insight into the molecular mechanisms responsible for the regulation of cell production and debate is being focused on the existence of pluripotent stem cells cable to originate neurons and Müller glial cells.

2. In most nerve centres, cell production is followed by a migrtion of immature neurons. The neurons and glial cells of the retina are generated in the scleralmost zone of the neuroepithelium, away from their definitive emplacement. During the first stages of development (E-4 to E-12), the retinal cells migrate following at least two patterns: the first involves migration, via translocation, of the nucleus through the body of the cell, which is atached to the limiting membranes of the retina either at its vitreous pole, or its scleral pole or both. This provides morphological informaton about the typology of the embryonic cell we are studying. Ganglion, bipolar, photoreceptor and Müller cells use this system of migration. The second type of migration is consistent with the proposal of Pasko Rakie during the seventies, according to which most of the neurons forming the organized nerve centres in layers, such as the brain cortex and the cerebellum, reach their definitive emplacement by slipping along axes pre-established by the radial glia. This migration model has been called gliophile and in the case of the retina was demonstrated for the first time by us (Prada et al., 1989), again reinforcing the notion that the processes of neuronal development follow generalpatterns in most of the zones and centres of the nervous system.

The radial glia, which arises very early on in the germinal neuroepithelium, has a transient lifetime and disappears towards the end of migration,

being converted into glial cells of the mature astrocyte type. Although very little is known about the cell mechanisms that regulate the identity and function of these cells, in recent years our team has been working on the nature and functional behaviour of Müller cells during retinal development.

Interneurons, horizontal cells and amacrine cells, most with a bipolar shape, released from the limiting membranes of the retina migrate following the glial columns formed by young Müller cells. We have studied this process experimentally and we have also analyzed the role of certain molecules considered to be morphogenic, such as GABA, during cell migration and axonal navigation in an attempt to gain a better understanding of the role played by certain molecules such as netrins and semaphorins, which seem to confirm the neurotropic hypothesis posited by Ramon y Cajal.

3. Once the neurons have attained their definitive emplacement, they differentiate morphologically, biochemically and functionally. The final end of differentiation is the formation of functional contacts among the neurons with a view to establishing neuronal circuits. This stage is undoubtedly one of the most interesting and the one that gives the finishing touch to development, coferring complete functionality to the neurons and glial cells so that they can develop their capacities and interactions as adult organs. Synapsis formation may last days or even weeks and arises from a cascade of complex molecular processes, most of them unknown. Synaptogenesis begins with a process of membrane recognition between pre- and post-synaptic neurons. During this process, neurons –as proposed by Roger Sperry with his "chemo-affinity" theory– use positioning molecules distributed in gradients that are complementary to those of their target neurons. In the chick retina, the synaptogenic process is very long, beginning at E-9 at the level of the inner plexiform layer and ending at E16/E16 at the level of the outer plexiform layer. Neuronal differentiation and contact act on other neurons and glial cells, leading to important changes in their molecular differentiation. An example is the union of photoreceptors in the outer layers of the retina with Müller cells. As from E-15, when synaptogenesis begins, these cells (according to a recent experiment carried out by us) induce Müller cells to secrete glutamine synthetase. This is a key enzyme in the metabolism of glutamate, which is functionally necessary in the photoreceptor-bipolar cell- ganglion cell pathway and possibly also as an alternative route in GABA synthesis. This shows, for the first time, how a developing neuron induces the molecular differentiation of an astrocyte, as Müller cells are considered to be. This study analyzed the chronology of the immunohistochemical expression of glutamine synthetase in all zones of the retina and, again, we observed that there is a gradient that coincides with those of neurogenesis, GABAergic expression and synaptogenesis.

We finish by stressing that the retina offers an excellent model for studying neuroglial interactions during development. It should be noted that we have seen that Müller cells, although classically considered to be astrocytes, behave functionally as ependymocytes, radial glia, astrocytes and oligodentrocytes. This is confirmed by our most recent studies carried out with specific glial markers such as antibody 3CB2 (Prada et al., 1995) and antibodies against enzymes present in astrocytes such as glutamine synthetase (Prada et al., 1998) and specific oligodendrocyte proteins (Prada et al., m.s. in preparation).

DEVELOPMENT OF THE URINO-GENITAL APPARATUS IN HUMAN EMBRYOS

J. Puerta Fonollá, F. Viejo Tirado, T. Vázquez Osorio and J. Murillo González

Department of Morphological Sciences I, Faculty of Medicine, Complutense University, 28040 Madrid, Spain.

The development of the urinary and genital systems has been studied in depth in different species, including humans. In all of them, the importance of correct nephritic development for the normal development of the genital apparatus has been highlighted.

The work of Wartemberg (1978, 1982, 1990) has revealed the participation of the mesonephros in the organization of the mesenchyma of the gonad, in particular the male gonad. The research of Satoh (1991) has described the involvement of the mesonephros in the organization of the female gonad.

In the present work we studied 40 human embryos, from 14 somites (stage 11 of O'Rahilly) up to 30 mm. VC (vertex-coccyx) (stage 23 of O'Rahilly). After embedding in paraffin, the embryos were sectioned serially at thicknesses between 7 and 10 μm on the different spatial planes. The sections were then stained with hematoxylin-eosin, azam, V.O.F. and Bielchowsky stain (in blocks).

The results suggest that the participation of the mesonephros in gonad morphogenesis occurs much earlier on than what was reported by Wartemberg and Satoh and, also, that this participation is not limited merely to the gonad; the mesonephros also participates actively in the formation of the paramesonephric duct.

The mesonephros contributes cells to the gonad throughout its development. The first cells become integrated in the celomic epithelium covering the region where the gonad later forms in phases in which the gonad is not even outlined. When the basal membrane of the celomic epithelium is lost, both cell types –celomic and mesonephric– are invaginated to form the stroma of the undifferentiated gonad. This developing gonad receives new cells from the mesonephros and this migration persists throughout the period analyzed.

In the development of the genital tracts we observed that the human embryo contains the structures described by Didier (1967, 1968, 1970) in birds to form the paramesonephric of Müller's duct.

Indeed, the celomic epithelium in the zone where the duct is later differentiated creates a ridge (mullerian crest) that becomes thicker and forms a plate (mullerian plate) that becomes invaginated to give rise to the paramesonephric duct.

In its descent to the urogenital sinus the paramesonephric duct receives cells from the mesonephric duct which, like a guide, provides cells until it reaches the posterior face of the urogenital sinus.

MYOSINS AND HAIR CELL DEVELOPMENT

K.P. Steel[1], T. Self[1] and S.D.M. Brown[2]

[1] MRC Institute of Hearing Research, University Park, Nottingham NG7 2RD, UK
[2] MRC Mammalian Genetics Unit, Harwell, Didcot, Oxfordshire OX11 0RD, UK

The deaf mouse, *shaker 1*, has a mutation in the gene encoding myosin VIIA (*Myo7a*). We have looked at the early development of sensory hair cells in the cochlea to uncover the role of this unconventional myosin in their development. In the normal mouse, the microvilli which cover the upper surface of the hair cells start to elongate and form a V-shaped array of tall stereocilia from around 16 days of gestation onwards. In mice with severe *Myo7a* mutations (*Myo7a*^{6J} and *Myo7a*^{816SB}), stereocilia start to form normally, with the correct orientation, but by 18 days there are signs of disorganisation of the regular bundle array. By 3 days after birth, the bundles are very disorganised, with clusters of stereocilia scattered around the top of the hair cell, often with the wrong orientation. However, the stereocilia do grow tall and do form clusters with rows of graded heights. The appearance of the cross links and the distances between adjacent stereocilia appear to be normal, examined by transmission electron microscopy. Myosin VIIA seems to be required for maintaining the correct organisation of the stereocilia bundle.

Posters

P. 1 ULTRASTRUCTURAL LOCALIZATION OF GLYCOCONJUGATES IN THE HATCHING GLAND CELLS OF THE TROUT EMBRYOS

M.I. Arenas[1], M.J. Blánquez[2], I. de Gaspar[2], B. Fraile and R. Paniagua[1]

[1] Department of Cell Biology and Genetics. University of Alcalá. E-28871, Alcalá de Henares, Spain
[2] Department of Anatomy and Embriology. Faculty of Veterinary Medicina. UCM

KEYWORDS: Fish, development, lectins, histochemistry, ultrastructure

INTRODUCTION: A histochemical, light and electron microscopy study of the hatching gland cells (HGCs), in incubated 50-day-old trout embryos is reported.

MATERIAL AND METHODS: The distribution of carbohydrate residues in the glycoconjugates of these cells was studied by means of a battery of 13 different lectins conjugated with horseradish peroxidase: PNA, ConA, LCA, WGA, SBA, UEA-I, HPA, DBA and digoxigenin: DSA, MAA, AAA, SNA and GNA. The identification of N- and O-linked oligosaccharides in the HGCs was performed by application of both chemical and enzimatic treatments.

RESULTS: In the present study, the presence of carbohydrate in HGCs have been studied by lectin histochemistry, with chemical and enzymatic treatments. The cytoplasmic granules of HGCs showed that sialic acid residues binded by SNA and MAA belong to N- and O-linked glycoproteins. DSA lectin showed a strong reaction, these galactose residues are syalilated. The fucose residues binded by AAA lectin are both N- and O-linked. Also, we have visualized mannose and galactosamine residues in the glycoproteins of cytoplasmic granules of HGCs.

DISCUSSION: From the results of this study, HGCs can be considered seromucous cells. These cells store high choriolitic enzyme (HCE) and low choriolitic enzyme (LCE) (Yamagami, 1996), but the cytoplasmic granules, the endoplasmic reticulum, and the Golgi complex, also contains other glycoproteins with residues of sialic acid, galactose, fucose, mannose and galactosamine present in N- and O-glycoproteins, which could contribute to modify environmental conditions (Strous and Dekker, 1992). The negative charge of these glycoproteins might be responsible for the rapide expansion of mucin to form a highly hydrated gel (Zancone, 1983) that facilites the action of these enzymes in programmed cell death and that plays a major role during the morphogenic events.

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P. 2 PHENOTYPIC CONSEQUENCES IN THE ANTERIOR CENTRAL NERVOUS SYSTEM DEVELOPMENT IN MOUSE CHIMERIC EMBRYOS CONSTITUTIVELY EXPRESSING *FGF4*

David Bueno[1], Helen Abud, Judith Skinner[2] and John K. Heath[2]

Department of Biomechistry, Univ. of Oxford, South Parks Road, OX1 3QU Oxford, Uk
[1] Dept. de Genética, Fac. Biología, Univ. de Barcelona, Avda. Diagonal 645, 08028 Barcelona, Catalunya (Spain)
[2] School of Biochemistry, Univ. of Birmingham, Edgbaston, B 15 2TT Birmingham, UK

Inductive interactions are fundamental to the development of multicellular organism. Among the different types of molecules involved, evidences have revealed that the Fibroblast Growth Factor (FGF) family has multiple roles during vertebrate embryogenesis. One of the approaches to the question of *fgfs* functions during mammalian development is to prepare chimeras between wild-type embryos and pluripotential embryonic stem (ES) cells harbouring regulatory mutations in the FGF system.

We obtained chimeric mouse embryos constitutively expressing FGF4 (gain of function). Cells of the ROSAb-geo11 cell line (with constitutive

expression of b-galactosidase) were electroporated with the PGKFGF4 (the entire mouse *fgf4* coding region under the control of mouse PGK-1 promoter) construct. Transformed ES cells were microinjected into the blastocoel cavity of C57BL6/J *Mus musculus* blastocysts, and they were transferred to the uteri of random bred MF1 *M. musculus* pseudopregnant recipients. The foster mothers were sacrified by cervical dislocation. Chimeric embryos were identified by staining of the embryo or the yolk sac for b-galactosidase. To explore the abnormalities, we analyzed whole embryos, sections stained in haematoxilin and eosin, and sections hybridized with *fgf4* and *shh* antisense riboprobes.

All chimeras which contained a substantial ES cell contribution (>50%) show anomalous development of the limb and anterior central nervous system (CNS). We center this communication in the anterior CNS development. Embryos older than 14-14.5 days post-coitum (dpc) showing phenotypic defects are never recovered. Most embryos at 10.5 - 12.5 dpc show absence of eye development, with the optic cups visible in the center of the head rather than contacting the surface ectoderm, and failure in neural tube closure in the midbrain area, exhibiting exencephaly, reduction of the diencephalon, and anomalous differentiation of the neuroepithelium. The craniofacial area, which derives from the neural crest, appeared normal. *In situ* hybridization analysis show that *shh*, which is ectopically expressed in the limb buds of the same embryos, is detected only in its normal expression areas.

Our results suggest that at least some interactions involved in eye development and CNS formation are disrupted by the activation of FGF-dependent signalling processes. The absence of *shh* ectopic expression, probably due to the absence of the correct FGF4 receptors and signal transduction pathways, indicates that this molecule may not be responsible for anterior CNS malformations (as appear to be the case for limb malformations). As FGF8 has been identified as an important signalling molecule for midbrain development, and several FGFRs can bind both members of the FGF family, we speculate that the reported malformations in the anterior CNS are due to some kind of interference between FGF4 ectopic expression and the receptors for FGF8.

P. 3 TISSUE-SPECIFIC DISTRIBUTION OF RETINOL BINDING PROTEIN RECEPTOR IN RODENT EMBRYOS CORRELATES WITH TISSUE SUSCEPTIBILITY TO VITAMIN A DEFICIENCY

Dominey Yallop, Laurent Antoni, Claes Båvik, David E. Ong and Simon J. Ward*

** Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield, UK*

The importance of vitamin A (retinol) and its biologically active metabolite, retinoic acid (RA) during mammalian development has long been established. Whilst total vitamin A deficiency (VAD) will not support pregnancy and excess RA is teratogenic in a number of species including man, less severe maternal VAD causes a well characterised series of malformations known as the fetal VAD syndrome.

In the cell, vitamin A signalling is mediated through the nuclear RA and Retinoid X receptors. These receptors are widely distributed throughout the embryo, including tissues unaffected by maternal VAD. It is likely therefore, that the control of vitamin A signalling does not reside entirely at the level of the nuclear receptors, but at the point of tissue-specific uptake of retinol and production of RA. A key protein in this control is the retinol binding protein receptor (RBPr). RBPr has been shown to mediate cellular uptake of retinol from systemic retinol binding protein (RBP) in mouse visceral yolk sac endoderm and human keratinocytes. In order to understand the tissue-specific control of vitamin A signalling, we have investigated the distribution of RBPr in developing rodent embryos by immunohistochemistry.

The results show the presence of the RBPr in extraembryonic tissues at presomitic-stages. RBPr can be detected in embryonic tissues as early as the 1-3 Somite Pair (SP) stage. By 8-12 SP, the RBPr is also found in many other tissues, including the sensory placodes, gut derivatives and heart. Other proteins involved in the generation of the RA signal (Cellular Retinol Binding Protein, Retinol and Retinal Dehydrogenases) are found specifically within these same tissues. These tissues are especially susceptible to conditions of VAD. This vould suggest that the RBPr is crucial for the uptake of retinol by the target tissues, facilitating tissue-specific generation of the RA signal. We are currently testing this in our laboratory.

P. 4 NEW INSIGHTS ON THE MEDIAL EDGE EPITHELIAL CELLS FATE DURING PALATAL FUSION

C. Martínez-Álvarez[1], C. Tudela[1], S. O'Kane[2], J. Pérez-Miguelsanz[1], J. Puerta[1] and M.W.J. Ferguson[2]

[1] Departamento de Ciencias Morfológicas I. Facultad de Medicina. Universidad Cumplutense. Madrid. Spain
[2] CID Division. School of Biological Sciences. University of Manchester. UK

Three mechanisms have been proposed to explain the disappearance of medial edge epithelial (MEE) cells during palatal fusion: cell death, epithelial-mesenchymal transformation (EMT) and migration to the oral and nasal epithelia. However, discrepancies have been reported regarding these processes. MEE cell death has not always been accepted as a real mechanism occuring during palatal fusion and its start point has not been well established. Similarly, labeling of MEE cells with vital lipophilic cell markers has not led to a clear conclusion on whether MEE cells migrate to the oral and

nasal palatal epithelia, transform to mesenchyme or both. The first aim of our work was to determine the onset and distribution of palatal MEE dead cells prior and during palatal fusion. By using the terminal deoxynucleotidyl tranferase-mediated dUTP nick end labeling (TUNEL) method on E14.5 CD1 mouse palates we found apoptotic dead cells in the MEE just prior to the contact and, profusely, in the midline epithelial seam throughout the fusion. Double labeling with TUNEL and F4/80 antibody showed the presence of macrophages close to the midline, but demonstrated that MEE apoptotic cells are also phagocytosed by adjacent F4/80 negative mesenchymal cells. Second, we tryed to ascertain whether MEE cells transform to mesenchyme during palatal fusion by infecting E13 MEE cells with the replication defective retrovirus vector CXL (Mikawa, 91) in culture. β-gal expression in TUNEL negative mesenchymal cells of post-fusion 4 days cultured palates led us to the conclusion that MEE cells do transform to mesenchyme. Finally, as TGF-β_3 has been shown to play an important role during palatal fusion, we wished to know the efects of the TGF-β_3 absence on MEE cells. We compared E13 to E15 TGF-β_3 null and wild type palates using Environmental Scanning Electron Microscopy, histological sections and TUNEL labeling. Our results showed many bulging cells on the MEE surface of E14 and E15 wild type embryos that were almost absent in the TGF-β_3 null clefted palates. These bulging cells are sites of initial contact between palatal shelves. However, apoptosis was not inhibited in the TGF-β_3 null embryos.

We conclude that MEE cells undergo apoptosis prior to the fusion and that both cell death and EMT are essential mechanisms to remove MEE cells from the midline epithelial seam. TGF-β_3 does not induce apoptosis during palatal fusion, but seems to act on the MEE cells cytoskeleton from E14, thus allowing these cells to adhere and, possibly, to transform to mesenchyme and to migrate.

P. 5 CLASSIFICATION AND DEVELOPMENT OF CLOACAL MUSCULATURE IN THE AVIAN EMBRYO

M. Forcada Jiménez, A. Peña Melián, J.C. Martín Rodríguez and A.J. Puerta Fonollá

Department of Anatomy of Madrid, University School of Medicine (UCM)

The development of the avian cloaca chambers has been studied very precisely, specially in *Gallus gallus*, but a little is known on the development of their miology. About the evolution of refered musculature it is possible to recognize in stages 34-35 of Hamburger and Hamilton (H.H.) (Hamburger & Hamilton, J.Morphol. 88, 1951) the mesenchyme not very differentiated which is enclosing cloacal chambers (Retterer, J. Anat. Physiol., 11, 1885). The outer muscular layer was observed at the beginning of stages 36-37 H.H. (Retterer, J. Anat. Physiol., 11, 1885), and finally the muscularis mucosae do not appear before stage 39 H.H. (Romanoff, The avian Embryo, 1960). Besides the only muscular structure appointed is the cloacal sphincter (Romanoff, The avian embryo, 1960). In contrast to this fault of reports about cloacal musculature in the chick embryo, there are more studies about this subject in the adult *Gallus gallus*. In the fowl the striated cloacal musculature, arranged all around the proctodacum, can be divided into two groups (Akita & Tatsuo, J. Morphol., 214, 1992): A) Sphincteric muscles group, which is integrated by two muscular portions: 1) m. sphincter cloacae and 2) m. transversus cloacae (this last member is double) and B) Levator muscles group, constituted by three paired and symmetrical portions: 1) m. levator cloacae, 2) m. pubocaudalis internus and 3) m. pubocaudalis externus. We have studied the development of the avian cloaca chambers using the habitual histological technics (H. E., azan stain,...) and the monoclonal antibody 13F4 which is specific for muscular cells (Pei-Ming, Peña Melián & Le Douarin, Dev.Biol., 122, 1987). Our results confirm the existence in the chick embryo, of similar muscular groups with its different members to the fowl, showing that the most important stages in the muscular development are 33 to 43 H.H. and the last muscles which finish their differentiation are the levator cloacae and transversus cloacae.

P. 6 ANATOMICAL RELATIONSHOPS OF THE ORBITAL MUSCLE AND THE CAVERNOUS SINUS IN HUMAN

J.F. Rodríguez Vázquez, J.R. Mérida Velasco, L.A. Arráez Aybar, J.A. Mérida Velasco, I. Sánchez Montesinos and J. Jiménez Collado

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INTRODUCTION: The orbital muscle of Müller is part of the orbital connective tissue system and represents and evolutionary vestige from earlier mammalian history. It was shown to be constantly present and to occupy a large part of the posteroinferior wall of the orbital cavity, extending cranially from the sphenoid bone and caudally to the zygoma and maxilla bones. The aim of this work was to study the associations between the orbital muscle and the cavernous sinus.

MATERIALS AND METHODS: Light microscopic studies were done on twenty human fetuses from the collection of the Institute of Embryology at the University Complutense of Madrid. The specimens ranged from 80 to 150 C-R length, corresponding to a gestational age of between 12 and 16 weeks based on Carnegie's stages.

RESULTS: Its upper surface is associated with the orbital content, especially with the inferior rectus muscle and the inferior branch of the oculomotor nerve. The orbital muscle in the human fetus

extends along the lateral wall of the orbit wich is not yet completely ossified and is connected to the lateral rectus muscle.The most important connections of the orbital muscle are those it makes with the infraorbital an zygomatic nerves.We observed how fibres of the orbital muscle proceeded dorsally across the superior orbital fissure and below the inferior ophtalmic vein to reach the inferior wall of the cavernous sinus, where the muscle fibres are isolated within adipose connective tissue that connects caudally with the pterygopalatine fossa.

DISCUSSION: According to our observations, the inferior ophthalmic vein lies on the upper surface of the posterior half of this muscle and some muscle fibres extend underneath this vein following its trajectory until it empties into the cavernous sinus in the inferior venous confluent.These muscle fibres are accompanied by orbital branches of the pterygopalatine ganglion that also lies along the lower wall of the cavernous sinus as decribed by Ruskell (J. Anat, 106; 1970).

P. 7 CRITICAL PERIODS IN THE PRENATAL MORPHOGENESIS OF THE HUMAN TEMPOROMANDIBULAR JOINT (TMJ)

J.R. Mérida Velasco, J.F. Rodríguez Vázquez, L.A. Arráez Aybar, J.A. Mérida Velasco, I. Sánchez Montesinos and J. Jiménez Collado

Dpto. de Ciencias Morfológicas II. Fac. de Medicina. Univ. Complutense. Madrid
Dpto. de Ciencias Morfológicas. Fac. de Medicina. Univ. de Granada. Granada

INTRODUCTION: Many studies have been published on the development of the human temporomandibular joint (TMJ), but different investigators disagree on its morphogenetic time table. Most discrepancies center on the moment of the initial organization of the condyle and the squamous part of the temporal bone and also the cavitation and onset of condylar chondrogenesis.

MATERIAL AND METHODS: Serial sections of 70 human specimens between week 7 and week 17 of development were studied by optical microscopy (25 embryos and 45 fetuses). All specimens were obtained from collections of the Institute of Embryology of the Complutense University of Madrid and the Department of Morphological Siences of the University of Granada.

RESULTS: Three periods in the development of the TMJ were identified: a) the blastematic stage (week 7-8 of development; O'Rahilly's stage 21 to 23), wich corresponds with the onset of the organization of the condyle and the articular disc and capsule. During week 8 (O'Rahilly's stage 23) intramembranous ossification of the squamous part of the temporal bone begins; b) the cavitation stage (week 9 and week 11 of development) corresponding to the beginning of formation of the inferior joint cavity (week 9) and the start of condylar chondrogenesis. Week 11 marks the initiation of organization of the superior joint cavity; c) maturation stage: after week 12 of development.

DISCUSSION: Our observations do not agree with those of Morimoto et al., (J. Prosth. Dent. 57, 1987), who described the stage of TMJ appearance between weeks 8 and 9, the preliminary stage of TMJ development between weeks 10 and 17, and week 21 as the end of TMJ stage completion.The blastematic and cavitation stages (7-11 weeks) constitute the critical period in the morphogenesis of the TMJ (Van der Linden et al. Am. J. Orthod. Dentofac. Orthop. 91, 1997).

P. 8 MESOTHELIAL CELLS MIGRATE TO THE VENTRAL AORTIC WALL IN AVIAN EMBRYOS

J.M. Pérez-Pomares, D. Macías, L. García-Garrido, R. Carmona, M. González and R. Muñoz-Chápuli

Departamento de Biología Animal, Facultad de Ciencias, Universidad de Málaga. 29071 Málaga, España

INTRODUCTION: The avian dorsal aorta shows a clear dorso-ventral asymmetry by E3 and E4. The ventral luminal cells are large, rounded and seem to constitute the earliest source of intraembryonic hemopoietic stem cells (HSC). The developing *tunica media* is much thicker in this ventral region, where the periaortic mesenchyme starts its differentiation into smooth muscle cells. We aimed to test a hypothesis forwarded by Olah et al., (Anat. Rec. 222, 1988) about a mesothelial contribution to the aortic wall which might to explain the transient asymmetry in the developing aorta.

MATERIAL AND METHODS: The sample studied consisted of quail embryos (*Coturnix coturnix)* staged between HH15 and HH24. The embryos were freed from extraembryonary membranes and fixed. Paraffin sections (histology and immunohistochemistry) and semithin sections were analized by light and confocal microscopy. Scanning and transmission electron microscopy were also performed.

RESULTS: By the stages HH16-HH22, the coelomic mesothelial cells adjacent to the aorta showed increased mitotic activity, reduced intercellular adhesion, loss of tight junctions, long basal cytoplasmic processes and cell overriding. These cells also displayed a distinct vimentin immunoreactivity. In these same stages, but not in later embryos, cytokeratin (CK) immunoreactive mesenchymal cells were found in the dorsal mesentery, ventral (but not dorsal) periaortic mesenchyme and aortic wall, even at adluminal levels. In a number of cells of the aortic wall, CK colocalized with smooth muscle cell (SMC)-specific α-actin and, occasionally, with the quail endothelial and hemopoietic marker QH1. The position of the HSC clusters coincided precisely with the areas of the ventral aorta located closer to the coelomic mesothelium.

CONCLUSION: We think that the CK and vimentin immunoreactive pattern observed in the periaortic areas can be explained by a translocation of cells from the coelomic mesothelium. These cells would undergo a cytoskeletal shift from an epithelial type (containing CK) to a mesenchymal type. The transient CK immunoreactivity, due to the CK remains in the mesenchymal cells, allows to track them in their invasion of the splanchnic mesoderm and aortic wall. The precise coincidence between the mesothelial contribution and the emergence of the aortic SMC progenitors and the intraaortic HSC clusters, as well as the immunohistochemical colocalization of antigens, suggests a potential relationship of the mesothelial-derived cells with one, or both, of these cell lineages. This may explain the observed ventrodorsal asymmetry in the distribution of SMC progenitors in the aortic wall, as well as the lateroventral position of the hemopoietic stem cell clusters.

P. 9 THE ORIGIN OF THE SUBEPICARDIAL MESENCHYME IN THE VERTEBRATE EMBRYO

D. Macías, J.M. Pérez-Pomares, L. García-Garrido, R. Carmona, M. González and R. Muñoz-Chápuli

Departamento de Biología Animal, Facultad de Ciencias, Universidad de Málaga. 29071 Málaga, España

INTRODUCTION: The primitive epicardium of the vertebrate embryo is originally constituted of a single cell layer that forms through spreading of splanchnic mesothelial cells over the myocardial surface. A space, the subepicardium, appears soon between the primitive epicardial cells and the myocardium, and is populated by mesenchymal cells whose origin was unclear. We suggest that a localized epithelial-mesenchymal transition involving the epicardium is the main source of subepicardial mesenchymal cells (SEMC) in the vertebrate heart.

MATERIAL AND METHODS: The sample studied consisted of dogfish embryos (*Scyliorhinus canicula*), quail embryos (*Coturnix coturnix*), chick embryos (*Gallus gallus*) and Syrian hamster embryos (*Mesocricetus auratus*). Dogfish embryos were anaesthetised with 0.04% tricaine metasulphonate before fixation. Pregnant hamster females were killed by chloroform overdose and the embryos obtained by laparotomy and fixed. The study included light microscopy, immunohistochemistry, scanning and transmission electron microscopy. Quail-chick chimeras were obtained through grafting of proepicardial tissue of HH17 quail embryos in the pericardial cavity of chick embryos.

RESULTS: The histomorphological study revealed clear similarities in the development of the SEMC in all the animal models studied. The atrioventricular junction (AVJ) and the outflow tract (OFT) were the earliest areas where a subepicardial space appeared between epicardium and myocardium. The epicardial mesothelium of the AVJ and OFT showed clear morphological traits of an epithelial-mesenchymal transition, i.e. loss of intercellular adhesion, cell hypertrophy and development of long basal cytoplasmic processes. In the transforming epicardial cells, as well as in the subepicardial mesenchyme, vimentin colocalized with cytokeratin, and the proteins fibrillin-2, ES/130 and the transcription factor Ets-1 were detected. On the other hand, proepicardial tissue of quail embryos grafted in the pericardium of chick embryos originated large patches of primitive epicardium which were morphologically identical to the host epicardium. The donor epicardium remained squamous on the atrium, but it transformed to mesenchyme in the AVJ and OFT. Donor-derived vessels and blood islands also developed in the subepicardium.

CONCLUSION: Morphological and immunohistochemical data suggest that the primitive epicardium of the vertebrates is a main source of SEMC through an epithelial-mesenchymal transition probably triggered by a regional-specific myocardial signal. The epicardial-derived mesenchyme probably contributes to the cardiac vascularization as well as to the development of the cardiac connective tissue.

P. 10 DEVELOPMENT OF *DE NOVO* BLOOD VESSELS IN PANCREATIC TISSUE TRANSPLANTS

E. Adeghate

Department of Human Anatomy, Faculty of Medicine & Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, United Arab Emirates

An intact vasculature linked to the host circulation is important for the survival and development of tissue transplants. The pattern of development of *de novo* blood vessels into pancreatic tissue fragments transplanted into the anterior eye chamber was investigated using india ink perfusion, light and electron microscopy techniques. New blood vessels from the host iris invaded the pancreatic tissue grafts within the first 24 hours of transplantation. These new blood vessels first appeared as sinusoidal blood capillaries which sprouted from the host iridal vessels to grow into the graft. Vascular anastomose between the encroaching iridal vessels and that of the surviving intrinsic blood vessels of the graft was established within 48-72 hours after transplantation. The micro- and ultrastructure of the blood vessels of the graft was intact even after several days of transplantation. Colored gelatin infused into the host blood vessels was discerned also in blood vessels of the graft. This observation demonstrated that vascular anastomose which led to the formation of host-graft circulation was established between the new blood vessels and that of the graft within 48 hours of transplantation. In addition to the intrinsic blood vessels of the graft, the endocrine and exocrine cells of the grafts survived with intact ultrastructure.

P. 11 ANGIOLOGY OF THE TERM PLACENTA OF THE RABBIT (*ORYCTOLAGUS CUNICULUS*) WHEN GESTACION OCCURS UNDER INDUCED ANEMIA CONDITIONS

G. ALexandre-Pires

Faculdade de Medicina Veterinária - Democ/ SEAN/Ciisa, Rua Gomes Freire, 1199 Lisboa Codex, Portugal

This research had in view the study of vascular alterations, caused by anemia conditions, as a cause of anoxia. An experimental model was developed. The original population of female rabbits was dichotomized into two groups: 1. The control group, which allow the definition of normal hematological values in this particular population; 2. the anemic population which was the group on which the author carried out clinical rehearsal, inducing hematological alterations (inoculation 3 times a week, into the marginal vein of the ear of 2 milliliters of (-hemolisin obtained from a *Staphylococcus aureus* filtrate –*Wood stock*– till the obtention of hemoglobin values between 8.6-9.4 g/dl), and whose animals were taken at random from the control group. According to the experimental protocol the females should become anemic before pregnancy and anemia should extend throughout the entire gestation. All procedures respected U.E. directives concernig the well being of the animals and experimental essays. The placentas studied were obtained through xyphopubic celiotomy of pregnant females, which were always anesthezided with natrium barbital. The research techniques were: 1. injection corrosion fluorescence technique; 2. injection- diaphanization; 3. histology; 4. scanning electronic mycroscopy in vascular moulds.

24 term placentas developed under anemia conditions were studied and a very particular vascular pattern can be described. The most striking feature when one compare this placentas with normal placentas is that the deciduous *cisternae* are replaced by arterial trunks of the basis of the decidua. These vessels start immediately their division process perforating the medular area of the placenta (venous deciduous area) in order to assume its typical cortical position where they divide into countless sprinkles of centrifugal/short capillaries. Contrarily to what is observed in normal placentas, in placentas from the anemic population , the use of a larger area of the maternoplacental *stratum* by small caliber vessels (the vascular «*puzzle*»), stands for an adaptation of the vascular bed of the maternal placenta to a reduction in oxygen saturated blood and a slow down in blood flow. Changes in the venous decidual system are a consequence of the reduction of available physical area because of the remarkable development of the arterial decidual system. The angioarchitecture of the vessels that stem from the umbilical arteries and vein remain similar when comparing normal placentas and placentas from the anemic population. Nevertheless an increase in the number of emergent vessels can be observed (the admirable system).

P. 12 DEVELOPMENTAL PATTERN OF NEUROFILAMENT EXPRESSION IN AUDITORY AND VESTIBULAR NEURONS. ROLE OF NEUROTROPHIN 3

I. San José[1,2], S. De la Fuente[1], R. Cabo[1], S. Rodríguez[1] and J. Represa[1,2]

[1] *Departamento de Anatomía Humana*
[2] *Instituto de Biología y Genética Molecular. Universidad de Valladolid, Consejo Superior de Investigaciones Científicas. C/ Ramón y Cajal 7, 47005 Valladolid, Spain. Fax: 34-983-423057*

Neurofilaments are the cytosketal intermediate filaments in neurons. They consist of three proteins (NFPs) with stimated molecular masses of 68, 150 and 200 kDa respectively. The expression of NFPs by cells is regarded as a hallmark of neuronal differentiation, particularilly during embrionic development. The precise knowledge of embryonal time in which the neuronal differentiation takes place, as well as of factors controlling this differentiation, particularily during embrionic development. The precise knowledge of embryonal time in which the neuronal differentiation takes place, as well as of factors controlling this differentiation, migh be of capital importance in experimental and/or pathologic studies in which NFPs are involved. Several aspects of cellular and molecular mechanisms of cytoskeletal changes leading to the neuronal differentiation remain still unknown. The present study was designed to analyze the temporal and spatial patterns of NFPs expression in the chicken cochleo-vestibular ganglion (CVG) neurons during embrional (from E4 to E18) and earliest posthatiching (P10 and P30) periods. We investigated the expression of NFPs in CVG neurons cultured in medium with neurotrophin-3 (NT-3 ranging between o.5 to 5 ng/ml concentrations. The presence of NFPs immunoreactivity (IR) was studied using both Western-blot, and immunohistochemistry on tissue sections and primary cultures. The 68 kDa NFP subunit was expressed from E4 to E10, 160 kDa NFP subunit was found in all embryonic developmental stages and at P10, and 200 kDa NFP subunit was observed from E10 to P30. The immunoreactivity for each NFP subunit detected in inner ear sections was consistent with the data obtained by immunoblotting. NFPs were localized in the neuronal perikarya and their processes which consented to establish the temporal pattern of innervation of the sensory neuroepithelia placed in the inner ear. Primary cultures matched the "in vivo" pattern of NFPs expression. Present results demonstrate that NFP subunits are developmentally regulated, suggesting that each of them may be specifically involved in the maturation of CVG neurons, especially in innervating their target cells. On the other hand, the findings obtained in cultured neurons, established a new relationship between neurotrophins and cytoskelatal proteins and claim for a regulation of NFP expression by neurotrophin-3. In summarym, we have demonstrated that NFP subunits are sequentially but not co-expressed and

that of all three subunits exits in the CVG neruons. Besides, CVG cultured neurons with NT-3 expressed NFPs in a pattern which matches the NFPs expression in vivo.

P. 13 RELATIONSHIP BETWEEN THE DEVELOPMENT OF THE STOMACH AND THE VAGUS NERVE

I. Sánchez-Montesinos; J.A. Mérida-Velasco, J. Espín-Ferra, S. García-Gómez*, J.F. Rodríguez Vázquez*, J.R. Mérida Velasco*, R. Barranco-Zafra, J. Jiménez Collado*

Department of Morphological Sciences, University of Granada
** Department of Morphological Sciences II, UNiversity Complutense of Madrid*

INTRODUCTION: The rotation of the stomach during its development is a subject of controversy: While some authors consider it to be only apparent, others believe there to be no doubt as to its occurrence. Analysis of the supposed or real rotation of the stomach is based on four main aspects: 1) unequal development of the stomach walls, 2) the shape of the "bursa omentalis", 3) the arrangement of the dorsal mesentery of the stomach, and 4) the organization and location of the vagus nerves. Although the first three of these morphological characteristics can be interpreted as negating the existence of gastric rotation, the fourth cannot.

MATERIALS AND METHODS: Forty-one human embryos were used in this study. Normal laboratory procedures were followed to prepara 10Ám-thick transverse or sagittal serial section, which were stained with hematoxylin-eosin for light microscopy study.

RESULTS: Our results confirm that the stomach acquires its definitive arrangement due to the joint effect of the following morphological facts: 1) the unequal growth of its walls, favouring the left-side wall and the dorsal mesentery of the stomach 2) the progressive development of the liver on the right-hand side, and 3) the organization of the "bursa omentalis".

DISCUSSION: We have compared our results with those of other autors in order to pinpoint the location of the polystratified gastric celomic epithelium, the arrangement of the dorsalmesentery of the stomach, the effect of the organization of the "bursa omentalis" in gastric morphogenesis, the reality or not of gastric rotation, as well as the distribution of the vagus nerves to shed light on the supposed gastric rotation. Our observations confirm that the gastric rotation is merely an apparent morphological phenomenon. The position and distribution of the vagus nerves in the stomach neither supports nor negates the rotation.

P. 14 IMMUNOCYTOCHEMICAL DEVELOPMENTAL PATTERNS OF THE THORACO-LUMBAR SYMPATHETIC CHAIN IN THE CHICK

J.A. Mérida Velasco, I. Sánchez-Montesinos, J. Espín Ferra, J.F. Rodríguez Vázquez*, J.R. Mérida Velasco* and J. Jiménez Collado*

Department of Morphological Sciences, University of Granada
** Department of Morphological Sciences II, University Complutense of Madrid*

INTRODUCTION: Chromaffin cells synthesize, store and secrete a complex mixture containing amines, structural proteins, enzymes and neurohormonal polypeptides. The vast majority of studies have been performed on mammals and only a few recent ones have dealt with avian species, focusing mainly on somatostatin, neuropeptide Y, and vasoactive intestinal polypeptide. The blending of this cocktail, apparently could be modulated by neural and hormonal signals.

MATERIAL AND METHODS: The immunocytochemical development of the thoraco-lumbar sympathetic ganglia in the chick and its adrenal counterpart, was studied from $3^{1/2}$ to 12 days of incubation, using antibodies to 17 separate antigens, including antibodies to pan-neuroendocrine markers, catecholamine synthesizing enzymes, proprotein-processing enzymes, and neuropeptides.

RESULTS: Some of the antigens studied were heavily expressed from the first days of development, e.g., Go protein-α subunit, thyrosine hydroxylase, and galanin, while for others a strong heterogeneity both in number of immunoreactive cells and intensity of immunostaining was recorded at the different stages, e.g., chromogranin-A, chromogranin-B, 7B2 protein, proprotein convertase 2 and 3, secretogranin II, somatostatin, and dopamine-β-hydroxylase. The first immunoreactivities to appear at day $3^{1/2}$ where those for HNK-1, tyrosine hydroxylase, chromogranin-A, and chromogranin-B.

CONCLUSIONS: Two different patterns were found in the developmental thoraco-lumbar sympathetic ganglia. In the first, concerning chromogranine-A and B, Go protein-α subunit, tyrosine hydroxylase, and galanin, virtually all sympathetic ganglia cells were strongly immunostained from day $3^{1/2}$ onward. Except for HNK-1, chromogranine-A and B, cells immunoreactive for all the remaining antigens showed a steady increase up to the day 12. In the second, including 7B2 protein, proprotein convertase 2, and secretogranin II, full antigenic expression was reached in prevertebral sympathetic ganglia cells, within days 10. We observed differences between medullary and sympathetic ganglia cells immunoreactivities, with regard to proprotein convertase 3, somatostatin, dopamine-β-hydroxylase, and met-enkephalin.

Some of this work was supported by the Dirección General de Enseñanza Superior e Investigación Científica (Grant PM97-0172).

P. 15 TABLE PSXF (PREDICTIVE OF SYNDROME FRAGILE X) FOR MALES PATIENTS IDENTIFICATION TO ORDER LAB TEST FOR THE FRAGILE X SYNDROME DIAGNOSIS

A. Arce Rivas[1], M.ª Eugenia Navarro Espinoza[2], H.M. Acosta Valle[3]

[1] *Genetista Clínica y candidata a Maestra en Ciencias Médicas de la Fac. de Medicina de Mexicali, UABC*
[2] *Maestra en Ciencias Morfológicas y Coordinadora del Área Morfológica de la Fac. de Medicina, Mexicali, UABC*
[3] *M.C.© y Maestro de tiempo completo de Bioquímica de la Fac. de Medicina de Mexicali, UABC*

ANTECEDENTS: The Fragile X Syndrome is a common hereditary form of mentaly retarded associated to a Fragile site Xq27.3. It's caused due to amplification of repetitions of CGG in the FMR1 gen. There's a difficulty in classic phenotypic identification, because individuals with this syndrome, don't reveal specific physical characteristics. There's necessity for clinic identification of this syndrome, because it's having an increase incidence of 1:1000 to 1:1500 in males and the test for its diagnostic has difficult availability and high cost. It has been tried using "check list" to detect individuals with Fragile X risk and have more probabilities of positive results at the moment to order the diagnostic test and avoid the waist of resources.

METHODOLOGY: A table named PSXF (Predictiva del Síndrome X Fragil), has being created with 14 characteristics into 3 groups (phenotypics, neurobehaviorals, and familiar history of mental retarded). Each characteristic receive a value according to their frequency rate reported by several authors in Fragile X patiens and 25 was the maximal value. A cut line in 12.5 (50%) has being proposed to consider it to send the patient for a diagnostic test. It was applied to 85 students in Special Education Schools, whose ages where in between 3 to 35 years, and a blood test was taken for cytogenetic diagnostic first, and later verification by hybridization with DNA probe in Southern Blot. Each parent's patient in the study was informed of the procedures of this project through consent form.

RESULTS: From the 85 individuals, that were studied by cytogenetic, (in only one growth medium, with non folate, and less than 5% of fetal serum), five fragile sites were observed, three of them were corroborated by Southern Blot, and two of them were negative for this second instrument, whose diagnosed 12 males with Fragile X Syndrome. Macroorchidism was only presented in two Fragile X patients and in four of them, testicles where absent, and the other 6 were normal. The squared Chi analysis was used for determining the predominant phenotype in Fragile X, only showed significance in behavior data: perseverance language, poor contact to the eyes, short time attention and touch defense. Even though family history antecedents of mental retarded was presented in 8 patients with positive Fragile X, it was non significant and it was not found present in no Fragile X. Big ears were not significant, same thing with flat feet.

CONCLUSIONS: The PSXF table designed and proved in this study, is not descarted as useful for soliciting lab test for Fragile X Syndrome diagnosis, but it's required to continue with the study in a open poblation for detecting morphologic characteristics and conductual idiosyncratic of our poblation, since it has being proved that the characteristic data used by refered authors, don't identify individuals in risk of having this syndrome.

P. 16 DEVELOPMENT OF THE OMENTAL BURSA DURING THE HUMAN EMBRYONIC PERIOD: COMPUTER-ASSISTED MORPHOMETRIC ANALYSIS

J. Nebot, E. Macarulla and P.J. Fábregas

Unitat d'Anatomia i d'Embriologia. Departament de Ciències Morfològiques, Facultad de Medicina, Universitat Autònoma de Barcelona. 08193 Bellaterra, Spain. e-mail: j.nebot@blues.uab.es

The aim of this study was to analyze, with morphometric support, if there is correlation between the growth of the omental bursa and the gastric wall and mesenteries. This relation was evaluated by means of the degree of proportionality between the simultaneous changes of the omental and no omental variables. Thus, computer imaging techniques were applied on cross sections (systematic random selected) of a graded series of ten human embryos –from Carnegie stages 11 to 23– (Bellaterra Collection -Prof. Doménech Mateu). The volume of the omental bursa, the gastric walls and the gastric mesenteries was calculated applying the principle of Cavalieri and using PC-Draft Plus software. In order to know the wideness of the dorsal and ventral gastric mesenteries, in each selected cross-section, skeletonization of each mesentery was performed by an image-analyser and suitable software (Visilog 5). The final result of all the resulting line lengths of a mesogastrium was multiplied by the mean distance between two cross-sections, so an area of a skeletonized mesogastrium was obtained (Microsoft Excel 5.0). Between Carnegie stages 11 and 12, the initial omental bursa development was related with the increments of gastric mesentery areas. When pancreatico-enteric recess appeared (stages 12 to 13) the omental growth was related with the increment of the area of the mesogastrium dorsal, and the decrement of the volumes of the gastric mesenteries and the gastric right wall. Between stages 16 to 17 the mesogastrium ventral volume decreased, this was the latter reduction related with the omental bursa increase. From the stage 16 the omental bursa expansion was in relation with the growth of the stomach and/or its mesenteries. In conclusion: a) our results were consistent with the theory of the early growth of the omental bursa into the compound mesodermal

anlage of the primitive right gastric wall and the gastric mesenteries; b) from stage 16, the growth of the stomach and its mesenteries determine the omental bursa volume.

P. 17 CYCLOPIA IN A PIG (*SUS SCROFA DOMESTICA*): A CASE DESCRIPTION BASED IN AN AXIAL TOMOGRAPHYC (TAC) STUDY

J.G. Monterde, A. Diz, A.M. Galisteo, M.R. Cano and J. Ostos

Dept. Compared Anatomy and Pathology. University of Córdoba, Spain

Cyclopia is characterized by an absence of billaterality of the ocular structures, accordingly to a variable degree of joining of both orbits and eyeballs toward the sagital plane of the face. This malformation is the most important sign of holoprosencephaly, going usally accompained by a sustitution of the nose by a tubular structure named proboscis. Cyclopia has been described in most of species, although in domestic mamals is frecuently described in sheeps, due to the action of alkaloids present in pastures (*Veratrum sp.*).

Axial tomographies of the skull and face of a newborn cyclops pig were compared with other from a newborn pig and a terminal pig phoetus (90 gestation days). Cyclops pig face was dominated by a long tube-shaped proboscis, with nasalfossas at the end. An unique eye protruded in the middle line under the proboscis. Rudimentary eyelids limited laterally a few deeped orbita.

Axial tomography showed the bony basis of the proboscis, corresponding with a grooved osseus structure equivalent to the bones that limit the nasal cavity (*os incisum, os nasale and maxilla*). In this case only one cavity, with absence of nasal wall, and containing remainders of the palate and *ós vomer*. There was as well a total absence of structures related to the mandible or mouth.

The eyeball corresponded to two fused ophtalmic vesicles, although it was evident the presence of two fused pupils at the middle line; tomography did not make evident the presence of cristalline, and revealed a soft-tissue density.

The skull cavity was limited by circular walls of high bony density, being not appreciable another cavities of the head, such as sinus or middle ear cavities (*bulla timpanica*). The petrotimpanic part of the temporal bone contained large vestibulococlear vesicles.

The encephalum showed up an homogeneous density, with an apparent absence of development of the ventricular system. It was not appreciable the separation of the cerebrum into two hemispheres (Holoencephalic brain).

Under the skull, there is a transversal bony arch which can correspond with the mandibular arch, maintaining its primitive embryonic stage; some close bony pieces suggested the presence of a rudiment of *os hyoideum*.

Alterations described above agree with previous reports of this malformaton in other species. The great development of the proboscis probably is related to the anatomical characteristics of the face in this specie, where the rostrum protrudes notably with respect to the rostral end of the mandible.

P. 18 MORPHOLOGICAL STUDY OF THE DEVELOPMENT OF THE DILATADOR IRIS IN THE CHICK EMBRYO

M.C. Barrio-Asensio, J. Murillo-González, A. Peña-Melián and A.J. Puerta-Fonollá

Universidad Complutense, Facultad de Medicina, Departamento de Ciencias Morfológicas I, Madrid, España

The intrinsic ocular musculature in birds is striated. Previous studies have described both the origin of the dilatador muscle from the iridial outer epithelium and the formation of striated fibers. However, these studies have not established the onset of the muscular differentiation.

The ontogenic development of the dilatador iris has been studied by immunocytochemistry and standard staining on chick embryos from stage 25 HH to the time of hatching. We have used the monoclonal antibody 13F4, a highly specific marker of muscular cells. We have observed immunoreactive cells of the dilatador muscle throughout the iridial outer epithelium at the stage 38 HH, except in the area closest to the pupillary margin. According to other investigators, we found myotubes in the ciliary region of the iris which progress toward the pupillary margin. However, our results indicate that the early development of the dilatador muscle occurs simultaneously throughout the iridial outer epithelium.

P. 19 PRESENCE OF FGF-2 IN CHICK EMBRYO NEURAL TUBE FLUID

A. Gato, M.I. Alonso, J.A. Moro, P. Martín and E. Barbosa

The fluid in the embryonic brain cavity in early developmental stages, known as Neural Tube Fluid (NTF), has been said to be directly involved in the morphogenesis and growth of this anlage. We have previously described the complex protein composition of chick embryo NTF, which suggests that one of its components could be involved in the behaviour of the neuroepithelial cells which are in contact with it. In this study we aim to demonstrate the presence of Fibroblast Growth Factor Type 2 (FGF-2) in chick embryo NTF, and its involvement in neuroblast cell replication. From NTF samples of chick embryos ranging from stages 18 to 26 H.H., and extracted by means of microaspiration, proteins were separated by electrophore-

sis (SDS-PAGE) and FGF-2 detected by means of western-blot. Next, the biological activity of NTF FGF-2 was blocked in vivo by intracavity microinjection, at midbrain level, with anti FGF-2 antibody in 18 H.H. stage chick embryos, which were reincubated up to stage 23 H.H. Evaluation of DNA synthesis in the neuroblasts was carried out by means of intracardiac bromodeoxyuridine injection one hour before the end of embryo culture, with subsequent detection in histological sections with the antibody anti bromodeoxiuridine. Western-blot revealed FGF-2 in the NTF as a 52 Kd band which remained constant at all the stages studied and with no variation in its concentration. Intracavity in vivo blocking of FGF-2 does not seem to affect the morphological development of brain vesicles; however, it significantly reduces DNA synthesis in the neuroblasts, above all at fore and mid-brain level. FGF-2 presence in the chick embryo brain vesicles cavity at early developmental stages, and its influence on cerebral neuroblast replication, supports our hypothesis that embryonic brain cavity fluid at early stages of development may act as an important intercellular channel between neuroblasts in contact with it, and that this communication route could be involved in important processes such as the replication and/or differentiation of neuroblasts.

P. 20 DEVELOPMENTAL EXPRESSION OF THE GROUP III METABOTROPIC GLUTAMATE RECEPTOR mGluR4a IN THE MEDIAL NUCLEUS OF THE TRAPEZOID BODY OF THE RAT

I. Elezgarai[1], R. Benítez[1], J.M. Mateos[1], E. Lázaro[1], A. Osorio[1], J. Azkue[1], R. Kuhn[2], T. Knöpfel[3] and P. Grandes[1]

[1] Dept. of Neurosciences, Fac. of Medicine and Dentistry, Basque Country Univ., 699-48080 Bilbao. Spain
[2] Nervous System, Novartis Pharma Inc., CH-4002 Basel, Switzerland
[3] Riken Brain Science Institute, 2-1 Hirosawaz, Wako-Shi, Saitama 351-0198 Japan

A pre-embedding immunocytochemical method for light microscopy was used to study the postnatal development of the expression of the group III metabotropic glutamate receptor mGluR4a in the medial nucleus of the trapezoid body (MNTB) of the rat. Immunoreactivity for mGluR4a was localized in axonal endings wrapping the principal globular neurons in MNTB, known as calyces of Held. The percentage of calyces of Held immunoreactive for mGluR4a increased progressively from postnatal day 3 (PND3), showing the highest peak of labeled calyces by PND9. From this postnatal age on, a gradual reduction in the number of mGluR4a-immunopositive calyces of Held was observed, reaching the lowest level of labeled profiles in adult tissue.

The developmental expression of mGluR4a in calyces of Held correlates well with previous studies in young animals showing a modulation of synaptic neurotransmission by group III mGluRs at these giant excitatory synapses made on MNTB principal neurons. All these observations together might suggest that the expression of mGluR4a, mainly between PND7 and PND12, might be relevant in the maturation and modulation of synaptic transmission at the calyces of Held.

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P. 21 POSTNATAL DEVELOPMENT OF THE CONNECTIONS BETWEEN CLAUSTRUM AND NEOCORTEX IN THE RABBIT

C. Reblet, R.I. Blanco-Santiago, A. Alejo, J.L. Mendizábal-Zubiaga, I. Gutiérrez-Ibarluzea and J.L. Bueno-López

Department of Neurosciences. The University of the Basque Country, E-48940 Leioa, Spain

The dorsal claustrum maintains connections with many areas of the adult cerebral cortex being either reciprocal or claustrocortical only. As different from the rat, the rabbit's claustrum has reciprocal connections with areas 17 and 18. Less is known about the development of these connections. We placed DiI crystals on fixed rabbit brain occipital cortices at 18, 21, 25 and 28 gestational days (GD). We injected in vivo, CTb or Biocitin in the same places on different postnatal days. Except the brains of 18 GD fetuses, that were fixed by immersion in 10% neutral formalin, the rest of the animals were perfused transcardially with a solution of 4% paraformaldehyde in PB 0,1 M pH 7,6.

At 25 and 28GD, the corticoclaustral axons reached the claustrum but no cells were retrogradelly labelled at it, though many retrogradely labelled cells were already present in the cortex. This suggests that the claustrocortical axons had not sprouted enough inside the cortex at these ages. The animals injected at P0 and sacrified at P1 showed both anterogradely labelled axons and terminals and retrogradelly labelled cells in the claustrum. The retrogrsde labelling extended in most of the two rostrocaudal thirds of the claustrum but in the caudal part the labelled cells occupied most of their dorsoventral extent. During the postnatal development, the labelling decreased in both directions rostrocaudally and rostroventrally. From P13 the topographic order appeared and in P25 the adult pattern was defined and restricted to the caudal third of the claustrum. Our data suggest that the number of claustrocortical cells are reduced during the postnatal development or lose some collaterals as occurs in other areas of the nervous system. Supported by grants FIS 94/1684 and UPV96-EB179).

P. 22 EFFECT OF TRANSPLACENTAL ADMINISTRATION OF ETHANOL ON LUNG DEVELOPMENT

M.ª G. Moreno Treviño[1], J. Sepúlveda Saavedra[1], N. López Serna[2], G. Arredondo de Arreola[3]

[1] Depto. de Histología, Fac. Med. UANL, Monterrey, N.L., México A.P. 1563
[2] Dpto. de Embriología
[3] Mirador de la Sierra#105 col. Lomas del Valle San Pedro Garza García, N.L. México

Alcohol ingestion during pregnancy causes lung hypoplasia, with deficient development, as well as an increase in lung diseases in newborns. In the present work we analyzed the histological and ultrastructural effects on the alveolar region of the lung of newborn rats that received transplacental administration of ethanol. 24 Sprague Dawley rats were divided intro three groups, receiving either a liquid diet with 5% ethanol, or liquid diet with dextrin-maltose as paired control, or rat-chow and water ad libitum as absolute control, from the beginning of gestation. Immediately after delivery, newborns were sexed, weighted, and the craneocaudal length determined. Products were anesthesized with ether, and lungs were obtained. A fragment of the upper lobe of the left lung was frozen, and the remaning embedded in epon. Thick sections were used for histological and morphometrical analysis and thin sections were prepared for the ultrastructural study.

Histological analysis showed that lungs from rats that received alcohol transplacentally had a diminution of alveolar spaces as well as thickening of the alveolar walls. In the alveolar linning besides the normal type I and II pneumonocytes, cuboidal light cells were found, that resembled type II pneumonocytes. They were PAS positive and at the ultrastructural level showed small isolated lamellar bodies, thus were identified as immature type II cells. Within alveolar walls there were cells with lipid accumulation as identified by a positive oil red stainning. At the electron microscope were identified as lipofibroblasts which normal occur in the lung before delivery, dissapearing for the 20 gestational day in the rat. Another finding was the presence of tubular structures lined by a columnar epithelium resembling the epithelial tubes present in early lung development.

To evaluate these changes, the number of light cells profiles, and lipofibroblasts profiles per 100 alveolar cavities was determined both in central and peripheral regions of the lung. Tubular structures were also counted in the same fashion. Results were expressed as mean values for each group and statistically compared with a T student test. In lung periphery light alveolar cells profiles and lipofibroblasts profiles were significatively increased in lungs from animals transplacentally treated with alcohol, while Type II penumonocytes were decreased in the periphery of the same lungs. Tubular structures were also more abundant in the lung periphery of alcohol treated rats.

With these results we can conclude that 5% alcohol given transplacentally causes a decrease in the maturation of the lung in the rat, particularly at the periphery, characterized by the presence of tubular structures, abundant lipofibroblasts, cells normally not present at birth; and inmmature type II cells in the alveolus, while the mature type II pneumonocytes are decreased.

The observed delayed development in the lung, and the incomplete cytodifferentiation of type II pneumonocytes might be related to an inadequate production of surfactant. This condiction, if present in humans, could be related to the lung problems described in newborns whose mothers ingested alcohol during pregnancy. Presently we are analyzing quantitatively and qualitatively the surfactant system in the same model.

P. 23 CHONDROGENESIS IN REPAIR OF RAT KNEE ARTICULAR CARTILAGE FOLLOWING OPERATIVE DEFECTS

M. Espanha and A.I. Lopes

Lab. of Anatomophysiology, Faculty of Human Movement, Technical University of Lisbon

The purpose of this study was to investigate the healing potential of articular cartilage, using osteochondral defects and providing normal articular function postoperatively.

Fifteen male wistar mature rats were anaesthetised with sodium pentobarbital (0,75ml/100 g weight; ip). A medial parapatellar incision was done, the pattela dislocated laterally and an osteochondral defect was created in the medial condyle with a hand drill (0.85 mm ø), until bleeding was observed. The animals were kept in cages where they could move freely. After one month and two months the animals were euthanised with an overdose of pentobarbital sodium, the hindlimb was amputated and the knee joint was immediatly openned and disarticulated. The samples were fixed in 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer, pH 7.4, decalcified with 7.5% EDTA and processed for paraffin embedding. Histological sections were cut (4 µm) through the center of the repair tissue, perpendicularly to it's surface and stained with Safranin O. The nature of the predominant repair tissue (tissue that corresponded to more than 50% of the total repair tissue in the defect) was assessed according to the predominant cell type from sections stained with safranin-O (Todhunter et al., J. Orthop. Res. 11, 1993) and classified in three types: *fibrous tissue* (flattened or spindle shaped fibroblasts embedded in a poorly stainning matrix); *fibrocartilaginous tissue* (rounded cells in lacunae beginning to differentiate towards chondrocytes in a more basophilic matrix than that of the fibrous tissue); *hyaline cartilage-like tissue* (chondrocytes or rounded cells in lacunae - chondrones - comparable to the mature hyalin cartilage in a normally or nearly normally staining matrix with safranin-O).

Repair tissue emerged from the osseus zone, probably originated from bone marrow mesenchymal cells that transform into primitive fibroblasts (fibrous tissue in 62,5% of the defects) in the early stage of repair (one month). Those cells, latter (two months), by metaplasia originated fibrocartilage (70% of the defects) and hyaline cartilage. However this type of tissue was never the predominant one. The main histological features found in the repair tissue at one month were: (I) hypercellularity in the deep zone, (II) flow of tissue coming from the subchondral region diverging to the defect margins and (III) hyperthrofic cells in the deepest zone. At two months the repair tissue showed: (I) superficial layer in which flat and compacted cells were disposed longitudinally as well as the collagen fibrils, (II) differentiation of the repair tissue in different types of tissues, both at cartilaginous and osseus level . A natural sequence of the repair process was confirmed by different histological features observed at one and two months. The environment conditions provided, seems to determine metaplasia of reparative cells towards fibroblasts in the beginning and after in fibrocartilaginous cells, chondrocytes and osteoblasts synthetizing typical matrices in their appropriate times and locations. Evidence of synthesis of fibrocartilaginous and cartilaginous matrices was defined by safranin-O staining. The different response of the reparative cells in the chondral and osseous regions of the same defect might be ascribe to a different ambience. Joint motion acts as a mechanical stimulus that can modulate the reparative cellular potential by physical transduction mechanisms or improving the nutrition by cycle loading and therefore the transportation of substances of high molecular weight, like growth and differentiation factors and autacoid into the tissue matrix.

P. 24 DIFFERENTIATION AND PROLIFERATION-RELATED ROLES OF FIBROBLAST GROWTH FACTOR RECEPTOR 1 AND 2 SIGNALLING IN THE DEVELOPING MOUSE SKULL

S. Iseki and G.M. Morriss-Kay

Dept. of Human Anatomy and Genetics, University of Oxford

Fibroblast Growth Factor Receptors (FGFRs) are required for skeletogenesis, mutations in FGFR1, R2 and more rarely R3 are associated with craniosynostosis (early fusion of the skull sutures). We are investigating the functions of FGFR signalling during development of the coronal suture in mouse fetuses. We previously reported that Fgfr2 is expressed in proliferating osteoprogenitor stem cells within the sutures, and the osteogenesis-related gene osteopontin is expressed in the differentiating preosteoblasts and osteoblasts; the ligand Fgf2 is present at high levels in the differentiated region and low levels within the suture (Iseki et al., Development 124, 1997). Here we show that Fgfr1 transcripts are expressed in differentiating preosteoblasts and osteoblasts, in which osteopontin and osteonectin are also expressed. Implantation of FGF2-soaked beads onto the sutural region (exo-utero surgery under Hypnorm anaesthesia) induces Fgfr2 down-regulation within 4 hours. Fgfr1 and osteopontin are up-regulated by 6 hours, with subtle upregulation of alkaline phosphatase but with no effect on osteonectin. By 24 hours, both alkaline phosphatase and osteonectin transcripts are decreased in the bead implantation area, while up-regulation of osteopontin and Fgfr1 is maintained. These results suggest that Fgfr1 signalling plays a key role in the regulation of osteogenis cell differentiation while Fgfr2 signalling is required to maintain the stem cells in a proliferative state. Increased receptor signalling stimulated by FGF2 favours differentiation of the stem cells. This is part of the normal mechanism of osteogenesis at the edge of the differentiating membrane bones, but the rate of differentiation can be enhanced by increased signalling both by the experimental addition of FGF2 and by FGFR mutation, leading to premature loss of the sutural stem cells.

P. 25 INMUNOHISTOCHEMICAL ANALYSES OF ABDUCENS MUSCULATURE DEVELOPMENT OF EYE IN THE CHICK EMBRYO (*gallus gallus*)

C. Maestro, J. Pérez-Miguelsanz, C. Martínez-Álvarez, J.C. Martín-Rodríguez and J. Puerta

Departamento de Ciencias Morfológicas I. Facultad de Medicina. Universidad Complutense. Madrid, Spain

The presence of methameric structures at cephalic level, like the origin of the extraocular muscles, is been debated actually. We studied the abducens musculature onthogenesis from the begining of the myogenic precursors until muscular masses development, between stages 9 and 36 HH (stages according to Hamburger to Hamilton, 1951) based on the high specificity of the 13F4 antibody (Rong et al., 1987) for an epitope which is present in the Z muscle bands.

The mesenchyme condensation is first stablished at stage 18 HH in dorso-caudal region of the paraxial mesoderm. At S-19-HH, appear 19-HH the first positive cells to the antibody, which are reached some hours later by axonal fibers of the VI craneal nerve, at that moment, the myoblasts fuse between them and form myocytes.

When the axons of the abductore nerve are divided into two branches (stage-25), the abducens musculature primordia segregates into two portions, the most craneal portion represents the common anlage of the pyramidalis nictitans and quadratus muscles, meanwhile the most caudal portion is recognized as the lateral rectus muscle, these fin-

dings indicate that the segregation of myogenic populations is initiated when an interaction between the motor axons and myoblasts occurs.

We do not believe that the dorsal obliquus muscle derivates from that condensation, like it is considered by others authors, also, we have not seen any relationship between the precursor condensation of the abductore musculature and the named intermediate mass.

Rong et al.: Dev. Biol. 122, (1987).

P. 26 TRANSFORMING GROWTH FACTOR-BETA-1 EXPRESSION IN THE MAXILLA AND MANDIBLE DURING POSTNATAL DEVELOPMENT AND GROWTH IN RATS

M.C. Manzanares, A. Rubert, R. Pérez-Thomas

Faculty of Dentistry, University of Barcelona, Spain

Growth factors are peptidic molecules whose essential functions are the regulation of the cellular proliferation and differentiation. Transforming Growth Factor-beta-1 (TGF-beta-1), first identified by Seyedin in the late 80's is synthesized by the osteoclasts and it represents one of the most influential factors in the bone formation processes.

The aim of our study is to describe the expression pattern of TGF-beta-1 in the maxilla and the mandible of growing Sprague-Dawley rats (*Ratus norvegicus)* in different developmental stages. The mandibles of 20 rats of 10, 12, 19, 21, 35, 45, 60, 75, 90 and 135 days were dissected after sacrifice by decapitation following chloroform sedation. The bones were decalcified in an EDTA solution during 10 to 30 days, and succesively included in paraffin and sliced in 7 microns-thick sections as for standard histological observation, then stained using antibodies against TGF-beta-1.

The results will be presented as microscopic pictures showing the immunoreactions visible in the growing areas of the bones studied: midpalatal suture, *symphysis menti*, mandibular body and mandibular condyle. TGF-beta-1 is widely expressed in the endochondral ossification areas (both primary and secondary), while the expression in areas of membranous ossification is scarce. *We conclude that TGF-beta-1 expression pattern changes with different growth stages and differs for the two different ossification processes.*

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P. 27 HOMOCYSTEINE AS AN ENVIRONMENTAL FACTOR THAT MIGHT PRODUCE NEURAL TUBE DEFECTS

M. Epeldegui, G. Valera-Moreiras*, J. Puerta, J. Pérez-Miguelsanz

Dpto. de Ciencias Morfológicas I. Fac. de Medicina. U.C.M.
** Universidad San Pablo-CEU. Madrid. Spain*

The neural tube defects (NTD) are very usual abnormalities. In Spain the prevalence is 3.13‰ (ECEM, 1998). THe etiology of NTD is genetic and environmental. It has been questioned that an environmental factor, as elevated levels of homocysteine (Hcys), is close related to NTD; because hyperhomocysteinemia was found in women who have birth of offspring with NTD (Eskes, 1998).

To study this hypothesis, we have used chicken embryos (*G. Gallus*) at stages (s) 3 to 10 HH, and after windowing the eggs and removing 1.5 ml of albumen, Hcys (20 μM) was added directly on the embryo. Embryos were reincubated until they reached s19-21, and they were fixed in Bouin's solution, embedded in paraffin wax, and serially sectioned (8 μm). The sections were stained with hematoxylin-eosin for morphological assessments.

Severe abnormalities were obtained when Hcys was added at s3 to 8 (50% of the survival embryos), which affected the head and the whole body, all of this made really difficult to know the exact stage. At stages older than 8 the severe abnormalities descended roughly (from 17% or lower). At s8+/9 an increase of NTD restricted to the spinal cord occurs. In relation exclusively with spinal cord defects, we could observe different kind of them: a narrowing of the central canal (the most common), open neural tube (spine bifida) and more than one neural tube. All of them are located at the lumbar region and they could appear together in the same embryo.

At s9/10 the majority of the survival embryos are normal (72%) and the spinal cord defects decrease (16%).

We think that NTD are directly related to the stage in which we applied this elevated dose of Hcys, which may modified the boundary between primary and secondary neurulation.

ECEMC. Serie IV, n.° 3, 1998.
Eskes, Nutr. Rev., 56, 1998.

P. 28 DISTRIBUTION OF THE ELASTIC AND OXYTALAN FIBERS IN THE CONNECTIVE TISSUE IN THE HUMAN FOETUS

A. Gerbino, A. Leone, L. Lipari and M. Buscemi

Institute of Histology and Embryology, School of Medicine, University of Palermo. Italy

INTRODUCTION: Despite the fact that the biological importance of the connective tissue in the morphogenesis has been repeatedly stressed in mammals, we are studying the relationship betwe-

en elastic fibers and Cathepsin D and Macrophages component in many development steps. The elastic system fibers include: elastic fibers, elaunin fibers and oxytalan fibers (Cotta-Pereira *et al,*. 1977; Ghadially, 1982). Our purpose in this work is to correlate the distribution of elastic and oxytalan fibers with distribution of Cathepsin D and Macrophages.

MATERIALS AND METHODS: Podalic segments of human foetuses at 12° week were fixed and embedded in paraffin. Serial transverse sections 10 µm thick to evidence the elastic fibers were stained with orcein and Weigert's resorcin-fuchsin methods. To visualize the oxytalan fibers the sections were stained with modified Weighert's method. Some sections were treated with antibody for Cathepsin D (Cathepsin D-estrogen-regulated-protein- monoclonal IgG2, *Ylem*) and some sections with human Macrophage LN-5 (IgM, *Ylem*).

RESULTS AND DISCUSSION: The observations of sections show that the oxytalan fibers form a close-mesh network in superficial dermal connective tissue, while a wide-mesh network in the deeper connective tissue. About the cartilaginous buds the fibers parallely running form really a dense fascia. In the same way, about the nerves these fibers are present in the epineurium, arrange about the external blood vessels and make a thick guaina closed to a single blood vessel. They, moreover, run in the perineurium and penetrate in the endoneurium, where surround, also, inter/intra fascicular vessels. The staining with orcein and Weigert's method reveals that the elastic fibers are in small number in the context of connective tissue, while are present about in the blood vessels and the epineurium. The immunohistochemical reactions show that Cathepsin D immunopositive cells are irregularly located and the Macrophages are numerous and uniformly located in the stroma, but particularly thickened about the walls of blood vessels. The distribution of elastic and oxytalan fibers in foetal stage is particularly interesting for the turnover of these components of the perichondral matrix and perivascular and perineural matrix. The immunoreactivity for Cathepsin D give a dinamic model of the mechanism evolutive processes non necessarily correlated to numerous Macrophages but also, probably, to processes of changes, and terminal formation of elastic system fibers during the morphogenesis.

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P. 29 HUMAN UMBILICAL CORD AND CHOLINESTERASES

A. Gerbino[1], M. Buscemi[1], B. Valentino[2], E. Farina Lipari[2] and A. Leone[1]

[1] *Institute of Histology and Embryology*
[2] *Surgical, Anatomical and Oncological Department, Medical School, University of Palermo. Italy*

INTRODUCTION: Large stromal area of human umbilical cord consisting of subamniotic stroma, Wharton's jelly and adventitia of blood vessels was histochemically tested to permit the visualization of a cholinesterasic reactivity (ChE), since on the innervation of umbilical cord the data are controversal (Spivach, 1943; Fox and Khong, 1990).

MATERIALS AND METHODS: The samples are fixed in Karnovsky (10 ml. Sodium Maleate buffer pH 6.0, 15 ml. CaCl 10% pH 7.0, 15 ml. Saccharose 0,8 M) at 4°C for 12 hours and then placed in Saccharose for 1 hour. After cryostat freezing -20°C, 20 µm thick sections were incubated in the Karnovsky medium for AchE-ChE demonstration (Martinez-Rodriguez *et al.,* 1964) in human umbilical cord of newborns.

RESULTS AND DISCUSSION: The distribution of cholinesterasic activity (AchE-ChE) in the stroma of human umbilical cord reveals no cholinergic nervous fibers in the gelatinous tissue and in blood vessels. A mixed reactivity for AchE-ChE is present in the tunica media of the blood vessels both arteries and veins (this reactivity is more intense in muscle circular layer), and in endhotelial cells. This presence can be related to the important role of endothelial cells in regulation of blood flow (Lüsher and Vanhoutte, 1990) also for the well documented presence in these cells of vasoactive peptide (VIP), substance P (SP), calcitonin gene-related-peptide (CGRP), natriuretic peptide (ANF) and arginine-vasopressin (AVP).

The absence of an innervation system gives to endothelial cells and myocytes a role of vasomotility to permit the important mother-foetus exchanges. The cholinesterases, important mediators of vascular permeability in foetus acting on smooth muscle tone, may play an interesting role on the histofunctional mechanism of umbilical cord. It must be recorded, moreover, as the myogenetic factors can take part in contractile function.

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P. 30 IMMUNOLOCALIZATION OF BRAIN MYOSIN-V IN THE EARLY DEVELOPMENT OF CHICKEN EMBRYOS

A. Azebedo[1], R.E. Larson[2] and L.O. Lunardi[3]

[1] *Departamento de Morfología, I.B., UNESP, Botucatu (SP)*
[2] *Departamento de Bioquímica, FMRP, USP, Ribeirão Preto (SP)*
[3] *Departamento de Morfología, FORP, USP, Ribeirão Preto (SP)*

This work shows the presence of brain Myosin-V during the first stages of the chicken embryo development. Myosin-V is a unconventional myosin recently characterized as a native protein, purified, sequenced, cloned and expressed. The blastula, gastrula and neurula embryos were processed "in toto" for the localization of Myosin-V using policlonal monoespecific primary antibody anti myosin-V and secondary antibody goat-IgG anti rabbit-IgG FITC-

conjugated. Preparations were examined with Confocal Microscopy. The embryos with 3 and 5 encephalic vesicles were sectioned and processed for the localization of myosin-V using primary antibody above described and secondary antibody goat-IgG anti rabbit-IgG peroxidase conjugated. These preparations were examined with light microscopy. The analysis shows that the blastula embryos present myosin-V immunoreactivity in the cytoplasm of epiblastic and hypoblastic cells; gastrula embryos present immunoreactivity in the cytoplasm of ectodermic, mesodermic and endodermic cells; and neurula embryos present strong immunoreactivity in the cytoplasm of neural border cells and slightly immunoreactivity in the ectodermic, mesodermic, endodermic, somitic and cordomesodermic cells. Embryos with 3 and 5 encephalic vesicles present strong myosin-V immunoreactivity in the cytoplasm of neural tube cells and slightly immunoreactivity in ever others embryonic tissues. These results suggest that myosin-V may be associated with cellular motility events during the development, through the interaction of the head and tail domains with the actin cytosqueleton and cellular membranes.

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P. 31 MORPHOGENETIC ANALYSIS OF *DOUBLEFOOT* MUTANT MICE: IDENTIFICATION OF A NEW MOUSE MUTANT AFFECTING THE *SONIC HEDGEHOG* SIGNALLING PATHWAY

C. Hayes, A. Haynes, P. Denny, M. Lyon, S. Brown and G. Morriss-Kay[1]

MRC Mammalian Genetics Unit and MRC Mouse genome Centre, Harwell, Didcot, Oxon
[1] Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford

The mouse mutant *Doublefoot (Dbf)* exhibits preaxial polydactyly of all four limbs in association with craniofacial defects. These defects are due to the ectopic and aberrant activation of the *Sonic hedgehog (Shh)* signalling pathway, as a result of the *Dbf* mutation. Here we describe additional developmental abnormalities in homozygous *Dbf* embryos, which are not recovered beyond the fifteenth day of gestation.

Dbf/Dbf embryos display defects not seen in heterozygous embryos, in particular, abnormalities of several organ systems where Hh signalling is known to play an important role, including the respiratory system. Mutatons in characterised elements of the Hh signalling pathway have been implicated in the genesis of basal cell carcinomas (BCC), a common human cancer of the skin. The skin of adult *Dbf* heterozygotes display hyperproliferative defects and shows increased density of hair follicles per unit area of skin, consistent with a possible oncogenic role for *Dbf*.

We have begun the positional cloning of the *Dbf* mutation. A large interspecific backcross of 846 progeny have been typed for markers in the civinity of the *Dbf* mutaton on mouse chromosome 1, and the *Dbf* locus narrowed down to a 0.47cM interval. We will report progress on the physical mapping of the *Dbf* non-recombinant region and the identification of candidate genes.

P. 32 EPIPHYSEAL CARTILAGINOUS CANALS IN TIBIA OF CHICK EMBRYOS

Wahid Y.M. Abd El-Aziz

83 Toman Bay St., Zeitoun, Cairo, Egypt

Fifty chick embryos of the white leghorn species were used in this work. The fertile eggs were kept in electric incubator at 37.2° C. The embryos were extracted at the ages of 11, 13, 14, 17 and 19 days. The upper end of their tibia were dissected and half of it kept in 10% formol saline and the other half kept in 2.5% glutaraldehyde and both groups passed through the usual steps for light and electron microscopic examinations. The purpose of this study is to analyze the development of cartilaginous canals in the tibia of chick embryos. It was found that the epiphysis of the tibia was invaded by branches of the perichondral blood vessels forming the cartilaginous canals at the 13 days embryos. The cartilaginous canals increased in size, branched and penetrated the epiphysis till the proliferating chondrocytic zone. Each cartilaginous canal contained an artery, and a vein in loose connective tissue, and surrounded by normal and vacuolated chondrocytes. We concluded that the vacuolated chondrocytes act as a phagocytic cells, engulfing the matrix and giving a way for the branching of the cartilaginous canals.

P. 33 PRE- AND POST-NATAL DIFFERENTIATION OF IMMUNOHISTOCHEMICAL EXPRESSION OF AROMATASE IN THE HYPOPHYSIS OF THE RAT

R. Vázquez[1,2], G. Vázquez[2], J. Carretero[1,2], M. Rubio[1,2], E. Pérez[3], E. Hernández[1] and E. Blanco[1,2]

[1] Laboratorio de Neuroendocrinología. Instituto de Neurociencias de Castilla y León. Universidad de Salamanca. Spain
[2] Dpto. de Anatomía e Histología Humanas. Facultad de Medicina. Universidad de Salamanca. Spain
[3] Dpto. de Cirugía. Facultad de Medicina. Universidad de Salamanca. Spain

Recently (Carretero et al., 1998), we have demonstrated the immunohistochemical expression of aromatase P-450 in the rat hypophysis. These findings suggest that aromatase may be modulated by gonadal steroids. The present study aims at analyzing the variations in aromatase expression that occur as from embryonic development up to old age in rats. Aromatase-immunoreactive cells were

found in hypophyses on day E17 prior to the expression of the gonadotropic hormones FSH and LH and other hypophyseal hormones such as GH or Prolactin. Expression was increased by day E19, accompanied by increases in the mitotic index and cellular numerical density and size (cellular and nuclear areas) with respect to E17. During embryonic and early post-natal ontogeny (d7, d14) no gender-related differences were found. The immunohistochemical expression of aromatase decreased in pre-puberal female rats (post-natal day 17) but not in males. Gender-related differences between female and male animals were observed in adult untreated animals. In female rats, no differences were found in the percentage of aromatase-immunoreactive cells along the estral cycle. By contrast, differences were observed in the reaction pattern; nuclear immunoreaction was observed in the proestrous and estrous phases and cytoplasmic reaction was found in diestrous phases I and II. The percentage of aromatase-immunoreactive cells was significantly higher in males than in females (34.40±2.93% vs. 0.84±0.22%, $p<0.01$). Contrariwise, in aged male rats the immunohistochemical expression of aromatase in the hypophysis had disappeared or was reduced to a very weak immunoreaction located in isolated cells, in some animals. These findings suggest that aromatase may be involved in the pre- and post-natal differentiation of hypophyseal cells and that it could play an important role in the regulation of the hypophysis by gonadal steroids in adult rats.

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P. 34 EVIDENCE BY *IN SITU* HYBRIDIZATION OF THE PRENATAL EXPRESSION OF Hox11 L1 IN DIFFERENT TISSUES OF THE FETAL MOUSE

J. Carretero[1,2], R. González-Sarmiento[1,3], R. Rodríguez[1,3], P. Delgado[3], F. Sánchez[1,2], M. Rubio[1,2] and R. Vázquez[1,2]

[1] *Instituto de Neurociencias de Castilla-León. Universidad de Salamanca. España.*
[2] *Dpto. Anatomía e Histología Humanas. Facultad de Medicina. Universidad de Salamanca. España*
[3] *Unidad de Medicina Molecular. Facultad de Medicina, Universidad de Salamanca. España*

The development of a complete animal from a single cell is the result of a series of mitotic divisions. The cells deriving from this proliferation follow a differentiation process through which they become specialized in certain specific functions. These differentiates cells are subject to a process of organization –embryonic segmentation– whose result is an animal composed of different but homologous units that become variations elicited by the expression of certain specific structural and regulatory genes. Each segment acquires a specific identity through the action of homeotic genes (Hox). The respective determining decisiones in this development depend on their expression or on the alternative removal of exons during the different stages of embryonic development. The Hox grouped-genes are involved in the activation of genes that lead to the segmental development of the embryo.

Another type of Hox genes also exists; these are dispersed throughout the genome and participate in the regulation of tissue differentiation and migration during embryonic development. Among them is the Hox11 L1 gene which, in mice, is located on chromosome 6 and has been characterized by homology with the Hox11 gene, initially described in a case of lymphoblastic leukemia. These genes are so called because they bear a sequence called homeobox that codes for a protein domain that binds to DNA. The genes would gherefore act as transcription factors. In particular, the Hox11 L1 gene has been associated with alterations in intestinal innervation, giving rise to the development of congenital hyperganglionary megacolon.

Using a probe specific to the mRNA of the Hox11 L1 gene, we have studied the expression of this gene using non-isotopic in situ hibridization in fetal mice during different stages of their embryonic development, mainly focusing our attention on periods of organ growth. Together with the sites already reported by other authors (deriving from the neural crest), we detected the expression of this gene during the development of the crystalline vesicle, the latter lens; during the development of the Wolff islets, in future hematopoietic cells, and during the development of the dermis, in future sensory receptors and structures close to the hair follicles. Although we have been unable to link expression to established malformations, our results suggest that owing to the nature of Hox genes these hitherto unpublished sites could be related to migratory disturbances of the neural crest, to the establishment of congenital cataracts or to the appearance of leukemia, as occur with the analogous gene Hox 11.