Title
Syndecan switching during mouse mandibular morphogenesis

Author
Van den Berg, Harry Robert

Publication Date
1996

Peer reviewed|Thesis/dissertation

SYNDECAN SWITCHING DURING MOUSE MANDIBULAR MORPHOGENESIS

by

HARRY ROBERT VAN DEN BERG

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

ORAL BIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date University Librarian

Degree Conferred: . . . . . .

To Lori

for being there

every step of the way

Preface

This research was conducted at the University of California, San Francisco as part of the advanced basic science training component of my Dentist Scientist Award K15 DE00365 from the National Institute of Dental Research, National Institutes of Health, Bethesda, MD. The advanced clinical science component consisted of the Postgraduate Program in Orthodontics at the University of California, San Francisco. This work was also supported by the "Willie and Earl Shepard Memorial Fellowship" provided by the American Association of Orthodontics Foundation. I hereby gratefully acknowledge these sources of support.

I would like to thank my mentor Caroline Damsky for her guidance and untiring support throughout my scientific training. I would like to thank Art Miller for his encouragement during this project and for his suggestions on the manuscript. I would like to thank Karin Vargervik for sharing her insight in the etiology, manifestations and clinical management of craniofacial malformations and for serving as a member of my thesis committee. I would like to thank Carles Bosch who's lectures during the first year of my training as an orthodontist helped inspire me to study the molecular basis of craniofacial development. I would also like to thank Nilda Ubana for preparing the paraffin sections and alcian blue stainings and Chanh Dinh for developing & printing many rolls of film. Thanks are also due to Jenny Chin for instruction in dissection technique, Zena Werb, Amr Moursi and Akitoshi Jikko for instruction in immunofluorescence methods and helpful discussions about the project.

Contents

List of Figures

Introduction

Craniofacial development

The specialty of orthodontics has a great interest in understanding the mechanisms involved in normal and abnormal craniofacial development. Clinical orthodontic diagnosis and treatment of a developing individual deal with an extremely intricate structure which is constantly changing, governed by tightly controlled and interactive mechanisms.

The importance of genetic and epigenetic factors in regulating craniofacial development is well understood. The spatial interactions that coordinate this process of pattern formation are ultimately controlled by the genes, however the exact mechanisms of the genetic control of body structure are largely unknown. Starting with the fertilized egg, detailed spatial signals - either intracellular or extracellular - are essential in providing cells with positional information to guide their specialization. An example of a molecule that can provide such a signal is retinoic acid, a known morphogen which affects anterior-posterior (head-tail) patterning along the axis of the early embryo and anterior-posterior (digits) patterning in the limb at a later stage in development (Jessel and Melton, 1992). The mechanisms that supply positional information in an embryo act over only small regions, morphogenetic fields, which comprise a limited number of cells (100 cells or less). The amount of detail that can be specified in such a morphogenetic field is limited. Therefore the final positional specification of a cell has to be built up as a sequence of items of positional information registered at different times. Cell memory therefore is crucial for the development of large complex organisms. Cells must not only become different, they must also remain different after the original cues responsible for cell diversification have disappeared. The cells' behavior is governed not only by their genome and their present environment, but also by their history. Moreover, a cells phenotype may lag behind its level of determination or

commitment, as specified by subtle controls at the level of the genes (Slavkin, 1990b; Alberts et al., 1994). It is therefore essential to characterize craniofacial development at the molecular level. Histological techniques such as in-situ hybridization, autoradiography and immunohistochemistry (Thesleff et al., 1991; Vainio et al., 1993) in combination with molecular biology techniques such as recombinant DNA technology, are expected to produce new answers to the regulation of craniofacial patterning and growth.

Considering the limited range over which positional cues act, the basic plan for craniofacial morphology has to be laid out very early in development, with continuous updates to specify additional levels of complexity. The initial organizational plan for the head is laid down at the time of germ layer formation through the process of gastrulation and neurulation. Experiments have shown that the mesoderm and notochord are the primary determinants of neural plate induction (Beddington, 1982). Further head development is dominated by the cranial neural crest. These cells form at the edge of the neural plate as ectomesenchymal cells and in the head they migrate under the surface ectoderm to populate the frontonasal, maxillary and mandibular processes. The neural crest cells that populate the first branchial arch emigrate from the rostral hindbrain at 8 days postconception (p.c.), beginning at the 5-somite stage and ending at the 10-somite stage, approximately 9 hours later. (Serbedzija et al., 1992). The cranial neural crest forms almost all of the skeletal and connective tissue of the face and anterior neck, the exceptions are the myoblasts of skeletal voluntary muscle and the endothelial cells of blood vessels, both of which are derived from mesoderm (Noden, 1991a and 1991b). The cranial neural crest also contributes to the odontoblasts, pigment cells, neural elements and it plays a role in cardiac septation (Kirby et al., 1983).

The craniofacial structures develop from five distinct primordia: the frontonasal process and the paired maxillary and mandibular processes. They form the face by regional growth and morphogenetic movements: the mandibular processes fuse in the midline; the medial and lateral nasal processes and the maxillary processes fuse and merge to form the nose, cheeks and the primary palate, separating the nasal and oral cavities and eventually forming the upper lip and portions of the anterior maxilla. The secondary palate is formed by fusion of the palatal shelves and terminal tissue differentiation (e.g. cartilage, bone, muscle) is initiated (Johnston and Bronsky, 1991a).

Abnormal development in the craniofacial region can take on many forms, such as isolated cleft palate, cleft lip and/ or cleft palate, macrostomia, hemifacial microsomia, holoprosencephaly. Certain genetic mutations, teratogens and environmental insults have been implicated in different craniofacial malformations. For example for several types of craniosynostosis (Crouzon, Pfeiffer, Jackson Weiss, Apert syndromes) and for achondroplasia, mutations have been found in fibroblast growth factor receptors (Shiang et al., 1994; Reardon et al., 1994; Muenke et al., 1994; Jabs et al., 1994; Wilkie et al., 1995). Retinoic acid is an effective agent for the treatment of cystic acne, but when used by the mother during the first trimester of a pregnancy is capable of producing severe craniofacial and oral clefts and limb defects in the fetus. Other well known teratogens are phenytoin (Dilantin), alcohol and cigarette smoking. Some of these factors are thought to affect neural crest cell function (Jones, 1990; Morris-Kay and Tuckett, 1991; Slavkin, 1996), however the exact mechanisms are largely unknown. Most evidence concerning the mechanisms of normal and abnormal craniofacial development is derived from experimental animals, in which the mechanisms appear to be virtually identical across the vertebrates studied (Johnston and Bronsky, 1991b). The mouse has been selected by several researchers as an experimental model. Experiments on mice have been done using

exposure to alcohol and retinoic acid, resulting in typical craniofacial malformations (Abbott and Pratt, 1991; Johnston and Bronsky, 1991b). Strains with mutations resulting in chondrodystrophia which leads to facial clefts and malocclusion have been identified (Brown et al., 1991). Genetically altered mice (transgenics) have been produced to study the elimination, mutation or overexpression of specific genes on development (Stephens et al., 1995; Satokata and Maas, 1994; Liu et al., 1995).

Molecules associated with craniofacial development

Current efforts in developmental biology are directed towards defining the timing and positional coordinates for molecular determinants which sequentially control embryonic development. Different categories of molecules are involved in this process: transcription factors, growth factors, growth factor receptors, structural proteins and structural protein receptors. The findings below indicate the high level of complexity in the interactions between these putative regulators of early embryonic development in general and in the craniofacial region in particular:

Retinoic acid (RA) is a known teratogen and has been shown to cause anterior to posterior transformation when administered to early mouse embryos. The otic pit is rostrally displaced in RA-treated embryos (Morris-Kay, 1993). Several reports of human teratogenous effects of retinoids have been presented. Some of the possible features are microcephaly, frontal prominence, hydrocephalus, hypotelorism, ear deformities, cleft palate, micrognathia and congenital heart defects. Both timing and dosage appear to affect the extent and severity of the malformations (Lammer et al., 1985; Granstrom et al., 1990; Abbott and Pratt, 1991). RA treatment of early mouse embryos leads to a more anterior expression of several Hox genes in the hindbrain (Wilkinson, 1993).

Homeobox genes are strong candidates for regulation of local patterning and differentiation in craniofacial development. This is evidenced by the recent discovery of a mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis (Jabs et al., 1993). Mice that express the mutated MSX2 gene or overexpress the normal gene also have a craniosynostosis phenotype (Liu et al., 1995). Mice with a constitutively expressed Hox-1.1 construct show craniofacial defects including cleft palate and malformation of the cervical vertebrae (Kessel et al., 1990). Several studies on how Hox genes function have concentrated on axis formation of the early embryo (Hunt and Krumlauf, 1992) and on formation of the limb. The distributions of homeobox containing proteins in the limb are suggestive of a directive role in the condensation pattern of pre-chondrogenic mesenchyme (Hall and Miayake, 1992). The expression patterns of homeobox genes in the early embryo have been shown to be modified in the anterior portion of the embryo by retinoic acid (RA) treatment (Morris-Kay, 1993), which further implicates them as key players in craniofacial patterning.

Mutations associated with growth factors (e.g. EGF, TGF alpha) and their receptors have been implicated in first branchial arch malformations including ablation of tooth and cartilage formation as well as retarded mandibular development (Slavkin et al., 1992; Shum et al., 1993). Mice with the EGF-receptor double knock-out (EGFR -/-) display craniofacial defects. They survive for up to 8 days after birth and suffer from impaired epithelial development in several organs, including skin, lung and gastrointestinal tract (Miettinen et al., 1995).

Cell to cell and cell to extracellular matrix (ECM) interactions participate in morphogenetic patterning during embryogenesis. The regulation required to coordinate the timing and position of developmental events in epithelia and mesenchyme may reside in the sequential

expression of cellsurface receptors for ECM molecules, e.g. integrins, syndecans (Trautman et al., 1991; Damsky and Werb, 1992), cell to cell adhesion receptors, e.g. N-CAM, cadherins (Hirsch et al., 1991; Jones et al., 1992; Kintner, 1992); and ECM molecules e.g. tenascin, fibronectin, laminin, collagens, thrombospondin (Adams and Watt, 1993). Such a regulatory scheme assumes that these molecules are expressed at defined times and positions during development. Indeed specific patterns of several of these molecules have been found in different stages of embryonic development (Trautman et al., 1991; Wood et al., 1991; Corless et al., 1992; Sutherland et al., 1991 and 1993). Matrix constituents and their receptors are known to interact with growth factors (Adams and Watt, 1993) and have been implicated as downstream targets of morphogens and homeobox gene products (Jones et al., 1992).

A biomechanical connection between ECM and intracellular components could represent a mechanism for signaling between cells and their environment during development. The sequential expression of receptors for ECM may regulate or represent different levels of patterning and differentiation. These concepts make the study of the syndecans, a family of transmembrane heparan sulfate proteoglycans (HSPG) of particular interest. Syndecans are known to associate with the cytoskeleton and are able to bind to ECM components, such as tenascin, fibronectin, thrombospondin and type I, III and V collagen, the fibrillar collagens. This binding is independent of calcium and magnesium ions and is abolished by trypsin treatment, thereby distinguishing it from matrix binding via the integrins only (Bernfield et al., 1992). Several findings indicate a potential role for syndecan-1 as a matrix receptor. Syndecan polarizes predominantly to the basolateral surfaces of simple epithelia in culture (Rapraeger et al., 1987) and to the site of initial matrix formation in early mouse embryos, at the interface between the primitive ectoderm and primitive endoderm (Sutherland et al., 1991). However, syndecan-1 also localizes to cell surfaces where there

is no matrix apparent, such as over the entire surface of stratified epithelia. On mesenchymal cells, syndecan-1 is located predominantly within the cells and not concentrated at adhesive sites. These findings indicate possible additional functions for syndecan-1 (Bernfield et al., 1992).

The syndecan family forms interactions with integrins, ECM ligands, growth factors and the cytoskeleton

Syndecans bind to many ECM molecules and growth factors in the extracellular environment via their heparan sulfate (HS) or chondroitin sulfate (CS) chains. These molecules in turn also bind to distinct cell surface receptors, such as specific integrins or a specific growth factor receptor. Syndecans could hereby participate in the formation of a receptor complex that is required for the ligand to generate its physiological action. Potential candidates for this dual interaction are EGF, FGF, thrombin, N-CAM and fibronectin, ligands that have a specific high affinity receptor, but that also bind to HS (Bernfield et al., 1992).

The integrins bind a variety of ECM components, many of which also bind to HS, such as fibronectin, laminin, vitronectin and type I collagen. Expression of integrin adhesion receptors is tightly regulated spatially and temporally and changes are associated with developmental transitions in the early mouse embryo (Sutherland et al., 1993; Damsky et al., 1993). A possible close association between integrins and transmembrane HSPGs has been shown in several experiments. Epithelial cells made deficient in syndecan-1 through stable transfection of an antisense syndecan-1 cDNA lose cell surface expression of E-cadherin and have reduced expression and altered distribution of β1 integrins. These clones grow as individual fusiform cells. In controls, growing as epithelia, syndecan-1 is associated with the zonula adherens: it co-localizes with the cortical band of actin filaments

and with E-cadherin and is closely associated with β1 integrins. Iherefore, syndecan-1 may maintain epithelial morphology and organization by acting as a matrix receptor and/or by organizing other adhesion molecules in a receptor complex (Kato and Bernfield, 1990) In confluent epithelial monolayers syndecan-1 is located on the baso-lateral surfaces of the cells (Bernfield et al., 1992).

Fibroblasts have been shown to attach and spread on the cell-binding domain of fibronectin but to not assemble focal contacts unless they interact with either the amino- or carboxyl-terminal heparin-binding domain of fibronectin (Woods and Couchman, 1992). Ihe α5 β1 integrin binds to the fibronectin RGD-containing cell-binding domain. Heparin and/or chondroitin sulfate-bearing transmembrane cell surface proteoglycans (syndecan family members, certain forms of CD44) and phosphatidylinositol-linked (glypican-like) proteoglycans are capable of recognizing the carboxyl-terminal heparin-binding domain of fibronectin (Bernfield et al., 1992). The informational content of fibronectin has been dissected extensively. The central cell binding domain supports cell attachment and spreading, but does not support focal contact formation. In addition, interaction of α5 β1 with just the fibronectin cell-binding domain appears to transduce a stimulatory signal for ECM remodeling by upregulating expression of metalloproteinases (Werb et al., 1989; Huhtala et al., 1995). The interaction of integral HSPG with the C-terminal heparin-binding domains of fibronectin as well as the cell binding domain results in signals that promote assembly of focal contacts, but does not reverse the stimulatory signal for matrix metalloproteinases. Interaction of the cell-binding domain plus the CS-1 region of fibronectin, which binds α4β1 integrin, does reverse this stimulatory remodeling signal (Damsky and Werb, 1992). The requirement for heparin binding can be circumvented by direct activation of PKC with phorbol esters. Syndecan-4 but not syndecan-2 is present in focal adhesions in rat embryo fibroblasts, human embryo fibroblasts, porcine retinal

pigmented epithelial cells and rat aortic smooth muscle cells. It co-localizes in focal adhesions with vinculin as well as with β1 and β3 integrin subunits. This suggests that this syndecan has a cooperative role with integrins in cell adhesion by helping to regulate cytoskeletal organization following cell attachment (Woods and Couchman, 1994).

Protein kinase C has been shown to phosphorylate the cytoplasmic domain of both syndecan-2 and syndecan-3 but not of syndecan-1 and syndecan-4 *in vitro*. This suggests that syndecan-2 and syndecan-3 are physiologic substrates of protein kinase C. The role of the phosphorylation may be to alter the interactions of the syndecans with primary receptors, such as the integrins, or with other components. The requirement for protein kinase C and syndecan-4 in the assembly of focal adhesions of fibroblasts does not seem to be based on the phosphorylation of syndecan-4 by protein kinase C (Prasthofer et al., 1995).

Syndecan-1 and integrin α5 β1 appear to associate physically in the absence of their ligand fibronectin. In mouse B-cell lines capping of syndecan-1 induces co-capping of integrin α5 and β1, but not of α1, α2, α4 and αv, each of which are expressed. Co-capping of β1 is less evident than that of α5, possibly because β1 forms complexes with several α subunits. Capping of CD44 does not induce co-capping of any integrin subunit. Capping of α5 does not co-cap syndecan-1, possibly because of its lower abundance. The co-capping is not mediated by syndecan-1 GAG chains or by cell surface fibronectin, since it is not prevented by enzymatic removal of GAGs, by adding exogenous GAGs, by adding GRGDS or by capping cells that produce HS-free syndecan-1 (Chun and Bernfield, 1993).

Syndecan-1 binds bFGF (Bernfield and Hooper, 1991) and cell surface heparan sulfate is thought to function as a low affinity receptor required for bFGF to bind to its high affinity

receptor and exert its effects (Yayon et al., 1991; Bernfield et al., 1992; Damsky and Werb, 1992). The high affinity receptors for FGF are present at the cell surface in lower abundance than the cell surface heparan sulfate proteoglycans. The more rapid turnover of cell surface heparan sulfate proteoglycans , as compared to the HSPG in the matrix, gives them the potential to regulate the activity of the FGFs by controlling their availability to the signal transducing receptor (Bernfield and Hooper, 1991). *In vitro* experiments with a monolayer of endothelial cells to which bFGF was added in a small area in the middle of the culture dish have shown that the radius of diffusion and action of bFGF is increased in the presence of heparin or soluble HSPG. The soluble HSPG may function as a carrier, or it may protect bFGF from proteolysis (Flaumenhaft et al., 1990). Different members of the FGF family seem to require specific heparan sulfate sequences. This introduces an additional level of control over growth factor action through the modification of glycanation of the syndecans (Elenius and Jalkanen, 1994).

Structural characteristics of the syndecans

The syndecan family is comprised of four related transmembrane proteoglycans. The name "syndecan" is derived from the Greek words "syndein" and "glychos", which mean "to keep together by binding" and "sweet", respectively . The amino acid sequences of the 4 syndecan family members deduced from cloned cDNAs show a high degree of sequence similarity, both between species and when comparing individual family members. They are all heparan sulfate (HS) containing proteoglycans, while syndecan-1 and -3 have been proposed to also contain chondroitin sulfate (CS) (Bernfield et al., 1992; Gould et al., 1992). Size, glycosaminoglycan (GAG) attachment sites and sequence indicate a closer structural relationship between the proteins of syndecan-1 and -3 (30% sequence identity, rat) and between syndecan-2 and -4 (38% sequence identity, rat) (Bernfield et al., 1992; Carey et al., 1992). They all have a highly conserved cytoplasmic domain, which has been

thought to associate with the actin cytoskeleton when cross-linked at the cell surface (Rapraeger and Bernfield, 1982). A recent study has shown that, in the case of syndecan-1, this association does not require the cytoplasmic tail (Miettinen and Jalkanen, 1994). The binding to the cytoskeleton may therefore be mediated through interaction of syndecan-1 with other molecules at the cell surface, such as the integrins. Every family member has a tyrosine internalization signal, which makes it likely that they could be rapidly internalized via coated pits (Bernfield and Hooper, 1991). The transmembrane domain is also highly conserved. Species comparison of syndecan sequences suggest that the extracellular domain is evolving extremely rapidly, except for the highly conserved GAG attachment sites and the protease-susceptible site near the plasma membrane. The GAG chains attach to serine residues of Ser-Gly pairs, which are usually flanked by acidic amino acids. Syndecan-1 may have variable amounts of chondroitin sulfate in addition to heparan sulfate chains. Syndecan-2, and -4 are thought to only contain heparan sulfate. Presence of chondroitin sulfate in syndecan-3 in addition to heparan sulfate has been hypothesized based on similarities in its GAG attachment sites to syndecan-1, but so far none has been found (Salmivirta and Jalkanen, 1995). Shedding of the extracellular domain is presumed to occur by cleavage at the dibasic protease-susceptible site, although the responsible protease is not known (Bernfield et al., 1992). Selective shedding has been shown to account for its absence on the apical surfaces and its accumulation at the basolateral surfaces of confluent epithelial monolayers (Rapraeger et al., 1986; Jalkanen et al., 1987).

Syndecan-1 shows tissue-specific glycosylation patterns. Three major isoforms have been found, which differ with respect to the number and size of the GAG chains. They are distinguished by their relative molecular mass: syndecan-1 from stratified epithelia and plasma cells is ~100 kD, syndecan-1 from simple epithelia and vascular endothelia is ~160 kD and syndecan-1 from cultured fibroblasts is ~300 kD. The different isoforms are found

at specific cellular sites: the smallest form surrounds cells, the intermediate form is at the basolateral cell surface and the largest form is predominantly intracellular. Furthermore, HS chains from murine epithelial (NMuMG), fibroblast (NIH 3T3) and endothelioid (BALB /c 3T3) cells show differences in size, distribution of heparinase cleavage sites and disaccharide composition (Kato et al., 1991). These differences in glycosylation can affect the binding properties of the proteoglycan, for example a form of syndecan-1 (with no or undetectable amounts of chondroitin sulfate) from tooth mesenchyme binds tenascin, whereas a form of syndecan-1 (with both chondroitin sulfate and heparan sulfate chains) from mammary epithelial cells does not (Salmivirta et al., 1991). These differences are assumed to be developmentally significant, however the mechanisms that regulate them are not understood (Bernfield et al., 1992). The syndecans show tissue specific distribution during development (Sutherland et al., 1991; Bernfield et al., 1992; Gallo et al., 1993; Kim et al., 1994) and in mature tissues (Trautman et al., 1991; Kim et al., 1994; Elenius and Jalkanen, 1994; Salmivirta and Jalkanen, 1995).

The syndecans bind to a wide variety of ligands: ECM components, cytokines, enzymes and enzyme inhibitors, transcription factors, viral coat proteins (Bernfield et al., 1992). This interaction has generally been assumed to be based on electrostatic interactions with the negatively charged, highly sulfated GAG side chains. It has been found however that hydrogen bonding, van der Waals forces and hydrophobic interactions play a major role in these interactions (Thompson LD et al., 1994; Thompson SA et al., 1994). A higher degree of specificity than originally anticipated is provided by "minimal' sequences: short (4-6 saccharides long) that seem to function as consensus sequences for binding (for review see Salmivirta and Jalkanen, 1995). Different family members of the syndecan family may bind to different ligands, for example syndecan-3 has been found to bind to FGF-2 but not to FGF-1, collagens and fibronectin (Chernousov and Carey, 1993).

Syndecan-1 binds to fibronectin and several different collagens. Also, the same type of syndecan may bind to different ligands based on its GAG chain composition. For example in a study on embryonic neural cell cultures it was found that a single 45 kD HSPG on day 9 of development binds to FGF-2 and not to FGF-1, while day 11 cultures bind to FGF-1 and not to FGF-2 (Nurcombe et al., 1993). Also the binding of syndecan-1 from different myeloma cell lines to type 1 collagen is based on its heparan sulfate fine structure (Sanderson et al., 1992 and 1994).

Syndecans and embryonic development

This study addresses the temporal and spatial distribution of a family of transmembrane heparan sulfate proteoglycans (HSPGs), the syndecans, in normal embryonic development in the mouse model. So far four family members have been identified: syndecan-1 (syndecan; Saunders et al., 1989a), syndecan-2 (fibroglycan; Marynen et al., 1989), syndecan-3 (N-syndecan; Carey et al., 1992; Gould et al., 1992) and syndecan-4 (amphiglycan or ryudocan; David et al., 1992; Kojima et al., 1992). Specifically, the role of the syndecans in the development of the mouse mandibular processes will be investigated. Several findings in the literature indicate the potential significance of the syndecans in this process. Syndecan-1 is first detected at the 4-cell stage mouse embryo. After gastrulation, syndecan-1 expression is strongest in the ectoderm and in the endoderm (Sutherland et al., 1991). Syndecan-1 has a patterned expression along the anterior-posterior axis in the mesoderm of mouse embryos in the gastrulating and neurulating embryo, at 7.5 to 8.5 days p.c., as evaluated using immunohistochemistry (Sutherland et al., 1991). It is at this time that important anterior-posterior information is determined. The pattern of expression in the early mouse embryo of syndecan-1 correlates with the potential patterning activities of FGF that have been observed during establishment of the antero-posterior and dorso-ventral axis in Xenopus (Amaya et al., 1993). Furthermore, the

syndecan-1 promoter has sites that appear to be characteristic for patterning genes, e.g. a binding site for the Antennapedia class of homeodomain-containing transcription factors (Hinkes et al., 1993).

Members of the syndecan family of heparan sulfate proteoglycans show distinct expression during subsequent mouse embryonic development (8.5 - 10.5 days p.c.), as evaluated using whole mount in situ hybridization. Syndecan-1 and -3 are expressed early, while syndecan-2 and -4 do not become apparent until at least 10.5 days p.c. Syndecan-1 and -3 are expressed at adjacent sites; e.g. in the neural folds, syndecan-1 is near the medial edge while syndecan-3 is lateral at the sites of neural crest emigration. In the first branchial arch syndecan-1 is in the peripheral mesenchyme while syndecan-3 is in the central core mesenchyme. Syndecan-1 and -3 also display sequential expression, for example at 8.5-9 days p.c. syndecan-1 is expressed by forelimb mesenchyme and somites while syndecan-3 shows no limb staining and is only in the anterior somitic region. At 10.5 days p.c. both syndecan-1 and -3 are in limb mesenchyme and along the entire somitic region (Gallo et al., 1993).

At different times in development, there is transient expression of different syndecan family members at sites of epithelial-mesenchymal interaction and at sites of mesenchymal condensation. In the development of the mouse limb, the intensity of immunofluorescence in the central core region decreases at 11 days p.c. and by 13 days p.c. the immunostaining is lost in regions destined for chondrogenesis and myogenesis. During *in vitro* culture of limb mesenchyme syndecan-1 expression is initially upregulated, but with further culture the antigen becomes reduced in chondrogenic foci and in association with myogenic cells (Solursh et al., 1990). There is no noticeable staining for syndecan-2 in the mesenchyme at the early morphogenetic phase, but the staining becomes notable with the onset of

chondrogenesis and with the differentiation of the dermis. During intramembranous ossification, for example in the mandible, staining is hardly detectable on the scattered mesenchymal cells, becomes pronounced once the cells aggregate in the osteogenic core and persists in the differentiating osteoblasts. As development progresses expression persists in the perichondrium, periosteum and in connective tissue cells. (David et al., 1993). In the embryonic chick wing syndecan-3 transcription is upregulated in areas of chondrogenic differentiation, as evaluated using in situ hybridization (Gould et al., 1992).

Syndecan-1 displays an intriguing pattern of expression during tooth formation, as evaluated with immunohistochemistry and in-situ hybridization. Syndecan-1 mRNA accumulates in the condensing mesenchymal cells around the invaginating epithelial tooth bud, which becomes more intense when morphogenesis advances to the cap stage. During the bell stage, when the cuspal pattern of the tooth is established, syndecan-1 mRNA transcripts are lost, and syndecan-1 is not expressed in terminally differentiated odontoblasts. In the epithelium, syndecan-1 is intensely expressed in the invaginating epithelial bud, but the expression is reduced during the cap and bell stages. An increase in syndecan-1 gene expression is seen in the pre-ameloblasts preceding their terminal differentiation into ameloblasts, which is accompanied by a complete loss of transcripts (Vainio et al., 1989 and 1991; Slavkin, 1991; Vainio and Thesleff, 1992a and 1992b).

The mesenchyme from the dental papilla is able to induce syndecan-1 expression in uninduced mesenchyme, as found in co-culture experiments. This has been interpreted as evidence that diffusible signals may mediate the induction (Vainio and Thesleff, 1992a). *In vitro*, a combination of FGF-2 and TGF-β (Elenius et al., 1992) results in increased syndecan-1 expression in mesenchyme derived NIH-3T3 cells. FGF-3 and TGF-β

(Heino, 1993; Vaahtokari et al., 1991) are expressed in the dental mesenchyme or in the epithelial bud. TGF-β alters the GAG composition of syndecan-1 (Rapraeger, 1989), which could mean that the affinity for certain ligands, such as growth factors could be altered. Therefore growth factor regulation could work at both the syndecan-1 expression level as well as at the composition of the syndecan-1 side chains (Salmivirta and Jalkanen, 1995).

Syndecan-1 expression during murine secondary palate morphogenesis is correlated with epithelial cell shape, packing and degree of differentiation, as evaluated by immunohistochemistry using monoclonal antibody 281-2 (Jalkanen et al., 1985). Initially, a simple cuboidal epithelium covers the palatal shelves, with uniform syndecan-1 staining on all their surfaces. Subsequently, the mid-oral epithelium becomes multilayered, the cells become more elongated and those adjacent to the mesenchyme lose staining for syndecan-1 on their basal surfaces. These same cells lose uniform syndecan-1 staining during the formation of a curvature in this epithelium, while maintaining punctate staining on their baso-lateral surfaces . Condensing mesenchyme immediately subjacent to this curvature becomes intensely stained for syndecan. Next, the architecture of the oral and nasal epithelia stabilizes with uniform syndecan-1 expression on all surfaces, except on their basal surface at the epithelial mesenchymal interface. The epithelial cells that form the midline seam of the elevated palate show a similar staining pattern at this stage, which is followed by either programmed cell death or the transition to a mesenchymal phenotype, which may be determined by their syndecan-1 expression (Brinkley et al., 1992). Studies with a polyclonal antibody directed against the syndecan-1 core protein indicate a loss of syndecan-1 staining of the medial edge epithelium, just prior to its transformation into fusiform cells (Fitchett et al., 1990). Thus, syndecan-1 may be involved in modulating

cell shape, architecture and fate during shelf reorientation and midline epithelial seam dissolution (Brinkley et al., 1992).

Several *in vitro* studies indicate the potential significance of syndecan-1 expression in mediating epithelial-mesenchymal transitions. Such transitions are known to occur during embryonic development, for example during tooth development (Vainio et al., 1991) and formation of the secondary palate (Fitchett et al., 1990). Experimental demonstration of a role for syndecan-1 in epithelial-mesenchymal transitions comes from studies in which a full length syndecan-1 cDNA in the antisense configuration under a beta-actin promoter was transfected into NMuMG mammary epithelial cells. This results in translation-inhibition of syndecan-1. Transfected cells with < 15% cell surface syndecan-1 display a fibroblastic phenotype, growing as individual fusiform cells. Control cells grow as islands of closely adherent cells. Therefore, reducing cell surface syndecan-1 alters the cell-matrix and/or cell-cell interactions required to maintain epithelial morphology (Saunders et al., 1989b). Conversely, transfection of a full length syndecan-1 gene into transformed mouse mammary tumor cells under a RSV-MMTV-LTR promoter results in high syndecan-1 expression and a well attached phenotype, despite treatment with androgen or glucocorticoid, which normally results in a change from an epithelial to a more fibroblastic phenotype in these cells (Jalkanen et al., 1990).

In the adult mouse syndecan-1 is expressed in epithelia (Hayashi et al., 1987) and also in leukocytes such as B-lymphocytes (Sanderson et al., 1989) and myeloma cells (Sanderson et al., 1992). Syndecan-2 is produced by mesenchymal cells (Marynen et al., 1989) and is particularly abundant in parenchymal organs like liver and kidney (Kim et al., 1994). Syndecan-3 is strongly expressed in neonatal rat brain and cardiac muscle while its expression in adult tissues is low (Carey et al., 1992). Syndecan-4 is expressed in the

avian nervous system and in muscle (Baciu et al., 1993). Syndecan-4 has been designated as amphiglycan because it is expressed in both epithelial and mesenchymal cells (David et al., 1992).

The following principles can be derived from these findings. Syndecan-1 seems to play a role in the early anterior-posterior patterning process in the embryonic mesoderm during axis formation (7.5 to 8.5 days p.c.). In epithelia its expression pattern suggests a role in maintaining epithelial morphology. During tooth formation and secondary palate formation, syndecan-1 is turned off in epithelial cells that undergo extensive morphogenetic activity and turned on in condensing mesenchyme. Syndecan-1 thus seems to be involved in the process of epithelial-mesenchymal transitions. Syndecan-3 is also seen in a patterned distribution in the early mouse embryo and may have a role in specifying patterning information at this stage. Later in development, syndecan-2 and syndecan-3 are seen in putative pre-chondrogenic mesenchymal condensations, while syndecan-1 becomes downregulated in these areas. Syndecan switching therefore seems to play a role in the local chondrogenic differentiation process. The distribution of syndecan-4 has been described during early avian embryonic development (Baciu et al., 1993). It is found in the developing nervous system, cardiac and striated muscle and in some epithelial tissues such as the lens and kidney. This distribution pattern is suggestive of a role in maintaining epithelial and neuronal tissue morphology, and also of a role in neurite outgrowth and myogenesis. Specific syndecans seem to have distinct functions. These putative functions appear to be dependent on syndecan-type, but also on the time and location of expression during development. The studies described in this thesis examine the distribution of individual syndecans during the morphogenesis and differentiation of the first branchial, in particular as they relate to the formation of the skeletal structures.

Hypothesis and specific aims

Hypothesis

Individual members of the syndecan family of cell surface proteoglycans display distinctive spatial and temporal patterning during the embryonic development of the mouse first branchial arch.

Specific aims

Characterization of the normal *in vivo* expression pattern of syndecan-1, syndecan-4 and fibronectin in the mouse first branchial arch in relation to:

1. Fusion of right and left mandibular processes
2. Fusion of mandibular and maxillary processes
3. Tooth formation, incisors and molars
4. Meckel's cartilage formation
5. Mandibular bone formation
6. Palatal shelf formation and elevation
7. Oral and buccal vestibulum formation

Normal whole mouse embryos allowed to develop in utero will be used to determine in detail the pattern of expression of syndecan-1, syndecan-4 and fibronectin in the first branchial arch, from 10.5 to 14.5 days postconception (p.c.). Specifically we will determine the correlations between the spatial and temporal expression pattern of these molecules and the 7 processes listed above.

During *in vivo* mouse development around 9.5 days p.c. the right and left mandibular processes fuse in the midline. Between 10 and 11 days p.c. the medial nasal process fuses

with the lateral nasal process and with the maxillary process to form the primary palate. Between 10 and 13 days p.c. the developing mandibular and maxillary processes fuse at their lateral borders to form the future cheeks and commissures. By 11 days p.c. incisor tooth organs are at late cap stages, whereas molars are at their initial stage of tooth development. Presumptive prechondrogenic ectomesenchymal cell condensations and molar tooth dental laminae are identifiable in the mouse mandibular process. By 12 days p.c. molar buds are evident in both the maxillary and mandibular processes. The lateral lingual swellings or presumptive tongue are well-defined in association with the forming mandibular process. Meckel's cartilage is evident by 13 days p.c. Mandibular alveolar bone formation occurs between 13 and 14 days p.c. At 13 days p.c. initial oral and buccal vestibulum formation is evident. Between 13.5 and 14 days p.c. the palatal shelves, which are initially directed vertically, elevate above the tongue starting anteriorly and fuse in the midline to form the secondary palate (Kaufman, 1992).

Preliminary experiments showed that syndecan-3 is predominantly expressed in the developing mouse brain, starting as early as 12.5 days p.c. (not shown). No antibody suitable for the detection of mouse syndecan-2 by immunohistochemistry was available at the time these experiments were performed. Within the syndecan family the structural characteristics and developmental expression patterns of syndecan-1 and -3 and similarly, of syndecan-2 and -4 resemble each other. This is indicative of the existence of two syndecan "subfamilies" (Salmivirta and Jalkanen, 1995). The subsequent studies feature the expression patterns of a representative from each subfamily, syndecan-1 and syndecan-4 respectively. The distribution of these syndecans is also compared with that of fibronectin, which interacts at least with syndecan-1 (Bernfield et al., 1992), and is a broadly expressed ECM ligand that plays a crucial role in cell migration and in the

differentiation of mesodermal cell types. Fibronectin has been shown to be closely involved with osteogenesis (Gronowicz et al., 1991; Moursi et al., 1996).

Materials and Methods

Specimen preparation

Timed pregnancies were obtained from Charles River Hollister, Hollister, CA. Mice were mated from 7-10 a.m., at which point they were separated. The embryos were harvested from 10.5 to 14.5 days postconception (p.c.). The embryo's were rinsed in PBS, placed in Bouin's fixative (Polysciences, Inc., Warrington, PA; 75 parts saturated picric acid, 5 parts glacial acetic acid, 25 parts 40% formaldehyde) for 2 hours, dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin.

Histology

Paraffin blocks were serially sectioned in the frontal plane, at 5 μm thickness. Representative sections were selected for immunohistochemistry or Alcian Blue staining. Alcian Blue specifically stains the chondroitin-4 and chondroitin-6 sulfate components of cartilage and was used to monitor cartilage formation. These sections were rehydrated, stained for 30 minutes with Alcian Blue (0.5% in 3% acetic acid), rinsed, stained for 5 minutes with Nuclear fast red, rinsed and coverslipped.

Immunohistochemistry

We used the rat anti-mouse monoclonal antibody 281-2 against syndecan-1. We used the rabbit anti-mouse polyclonal antibody MSE-4 against syndecan-4. The antibodies against syndecan-1 and syndecan-4 were kindly donated by Dr. Merton Bernfield, Harvard Medical School. The specificity of these antibodies has been reported before (Jalkanen et al., 1985; Kim et al., 1994). The 281-2 antibody recognizes the ectodomain of the core protein of syndecan-1. The MSE-4 polyclonal anti-serum recognizes the ectodomain of the core protein of syndecan-4. The polyclonal rabbit anti-mouse fibronectin antibody

A117 was purchased from GibcoBRL, Gaithersburg, MD. Secondary antibodies, normal goat serum and normal donkey serum were purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. Paraffin sections were rehydrated and incubated at room temperature with 10% normal goat serum in PBS to block non-specific staining. Primary antibodies against syndecan-1, syndecan-4 and fibronectin, diluted in PBS with 0.1% tween-20 and 0.2% bovine serum albumin, were incubated with adjacent sections overnight at 4 °C in a humidified chamber. The sections were rinsed for 1 hour in 5 changes of PBS with 0.5% tween-20. Fluorescein conjugated goat anti-rat (syndecan-1) or goat anti-rabbit (syndecan-4 and fibronectin) secondary antibodies, diluted in PBS with 0.1% tween-20 and 0.2% bovine serum albumin, were incubated with the sections for 1 hour at room temperature. The sections were rinsed for 1 hour in 5 changes of PBS with 0.5% Tween-20 (purchased from Sigma Chemical Company, St. Louis, MO). A drop of Vectashield^{R} mounting medium (Vector Laboratories, Inc., Burlingame, CA) was placed on each section to prevent quenching of the fluorescence signal and they were coverslipped.

For some of the sections the biotin-streptavidin technique was used. The blocking kit was purchased from Vector Laboratories. These sections were rehydrated and incubated at room temperature with 10% normal donkey serum in PBS to block non-specific staining. The sections were incubated for 15 minutes with avidin blocking solution #1, rinsed in 3 changes of PBS, incubated for 15 minutes with biotin blocking solution #2 and rinsed in 3 changes of PBS. The sections were incubated with the primary antibodies overnight at 4°C in a humidified chamber. Biotinylated donkey anti-rat (syndecan-1) and biotinylated donkey anti-rabbit (syndecan-4 and fibronectin), diluted in PBS with 0.1% tween-20 and 0.2% bovine serum albumin, were incubated with the sections for 1 hour at room temperature. The sections were rinsed for 1 hour in 5 changes of PBS with 0.5% Tween-

20. The sections were incubated with fluorescein conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted in PBS for 20 minutes. The sections were rinsed in 5 changes of PBS, a drop of Vectashield^{R} mounting medium was placed on each section and they were coverslipped.

The sections were viewed under a Zeiss Axiophot fluorescence microscope. Representative specimens were photographed using TMAX 400 film (Eastman Kodak Company, Rochester, NY). Controls utilizing non-immune serum and secondary antibody alone showed no staining (not shown).

Code numbers of embryo's illustrated in plates 1-6

Plate	Embryo code
1a,b	E10B052096.1
2a-d	E11B052196.1.1
3a-e	E12B050196.2
4a,b	E13B041596.1
5a-c	E13B041596.2
6a-c	E14B041696.1

Results

The expression patterns of syndecan-1 (S1), syndecan-4 (S4) and fibronectin (FN) in the developing mouse head, investigated from 10.5 to 14.5 days postconception (p.c.), are outlined below. The plates for each day of development are included at the end of the description of staining patterns that refers to them.

10.5 days p.c. (Plates 1a,b)

Head - General distribution (Plate 1a)

S 1 Predominantly in first branchial arch, maxillary as well as mandibular component, and lateral to developing eye.

S 4 Predominantly in neuroepithelium of developing brain and in ganglia and nerves.

F N Expressed in mesenchymal tissues, strong presence in basement membranes.

Maxillary process & roof of pharynx (Plate 1b)

S 1 Within maxillary process present in oral and dermal epithelium and mesenchyme. Epithelial expression slightly reduced in epithelial thickening of dental lamina, with moderate S1 expression in the underlying mesenchyme. Slightly reduced expression in band of mesenchyme underlying dermal epithelium. Very weak mesenchymal S1 expression within roof of pharynx.

S 4 Within maxillary process present in epithelium and throughout mesenchyme. Epithelial expression strongest in epithelial thickening of dental lamina. Mesenchymal expression strongest in medial part, within roof of pharynx.

F N Within maxillary process presence in medial part stronger than in lateral part. Closer to midline, within roof of pharynx, very weak FN expression.

Mandibular process (Plate 1b)

S 1 Within mandibular process expressed in oral epithelium and underlying mesenchyme; inferior dermal epithelium and narrow band of underlying mesenchyme. Reduced expression in median and lateral epithelium and mesenchyme, as well as in central transverse band of mesenchyme.

S 4 Epithelial expression strongest in midline area. Within mesenchyme of mandibular process a banded expression pattern is seen with strong expression in median mesenchyme and intermediate expression in lateral mesenchyme. Minimal expression in central core of right and left mandibular processes.

F N Within mandibular process a banded expression pattern is seen with moderate staining in the lateral mesenchyme, strong staining in the median mesenchyme and weak staining in the intervening area.

Plate 1a. Frontal sections through head of 10.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. At this stage of development the right and left mandibular processes have just fused in the midline. Significant growth has occurred in the developing brain, which appears disproportionately large as compared to later stages of development. Bar, 500 μm. ▬▬▬

1aa - first arch artery

cm - cephalic mesenchyme

dn - diencephalic neuroepithelium

f r/l - fusion right & left mandibular processes

mdp - mandibular process

mxp - maxillary process

os - optic stalk

tv - telencephalic vesicle

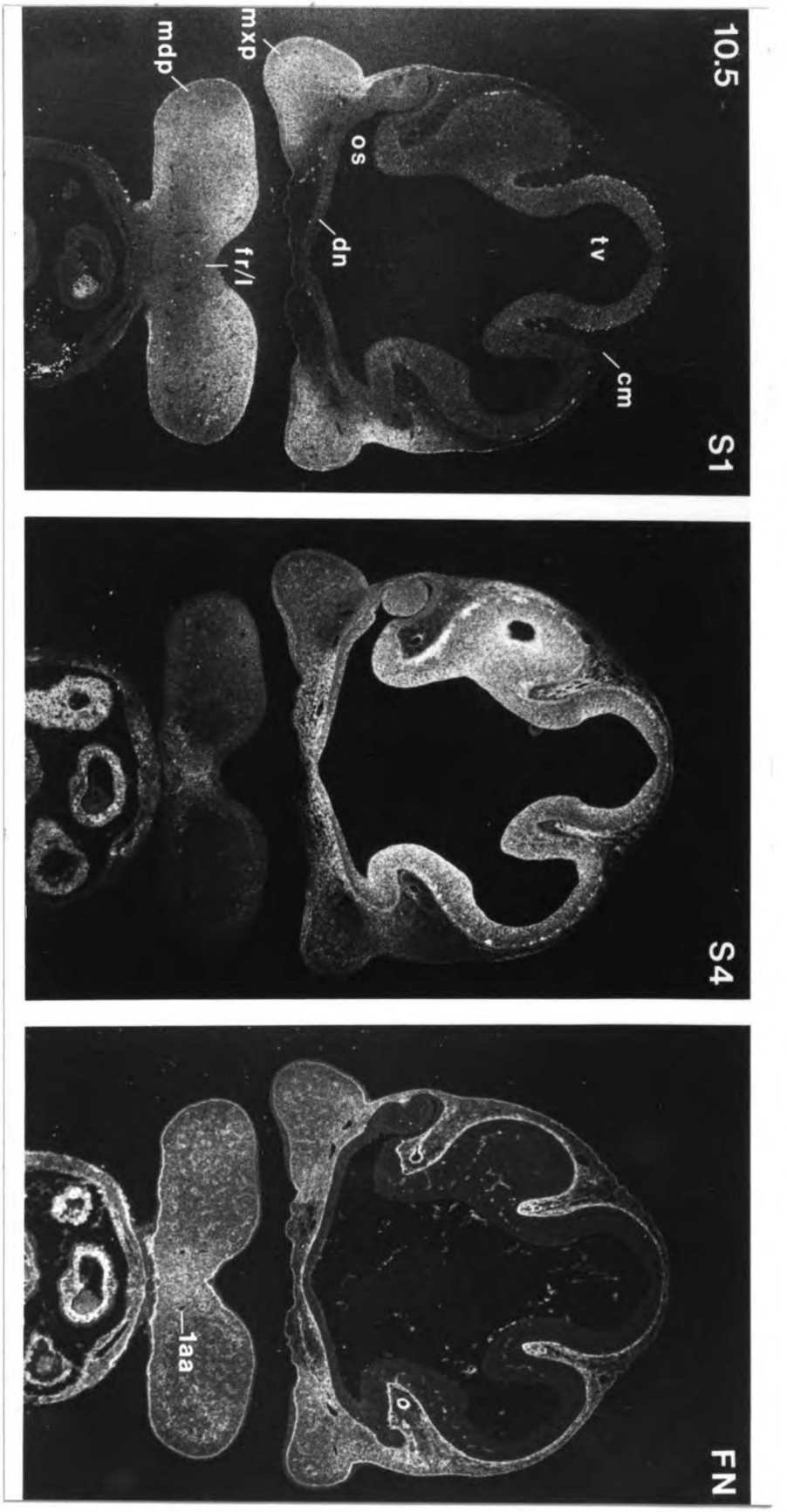


Plate 1a

Plate 1b. Same sections as plate 1a, close-up of maxillary and mandibular processes of 10.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. The fusion area of the right and left mandibular processes can be seen in the middle of the lower half of the images. In the maxillary process the developing dental lamina of the molar region is evident. Bar, 290 μm. ▬▬▬

1aa - first arch artery

de - dermal epithelium

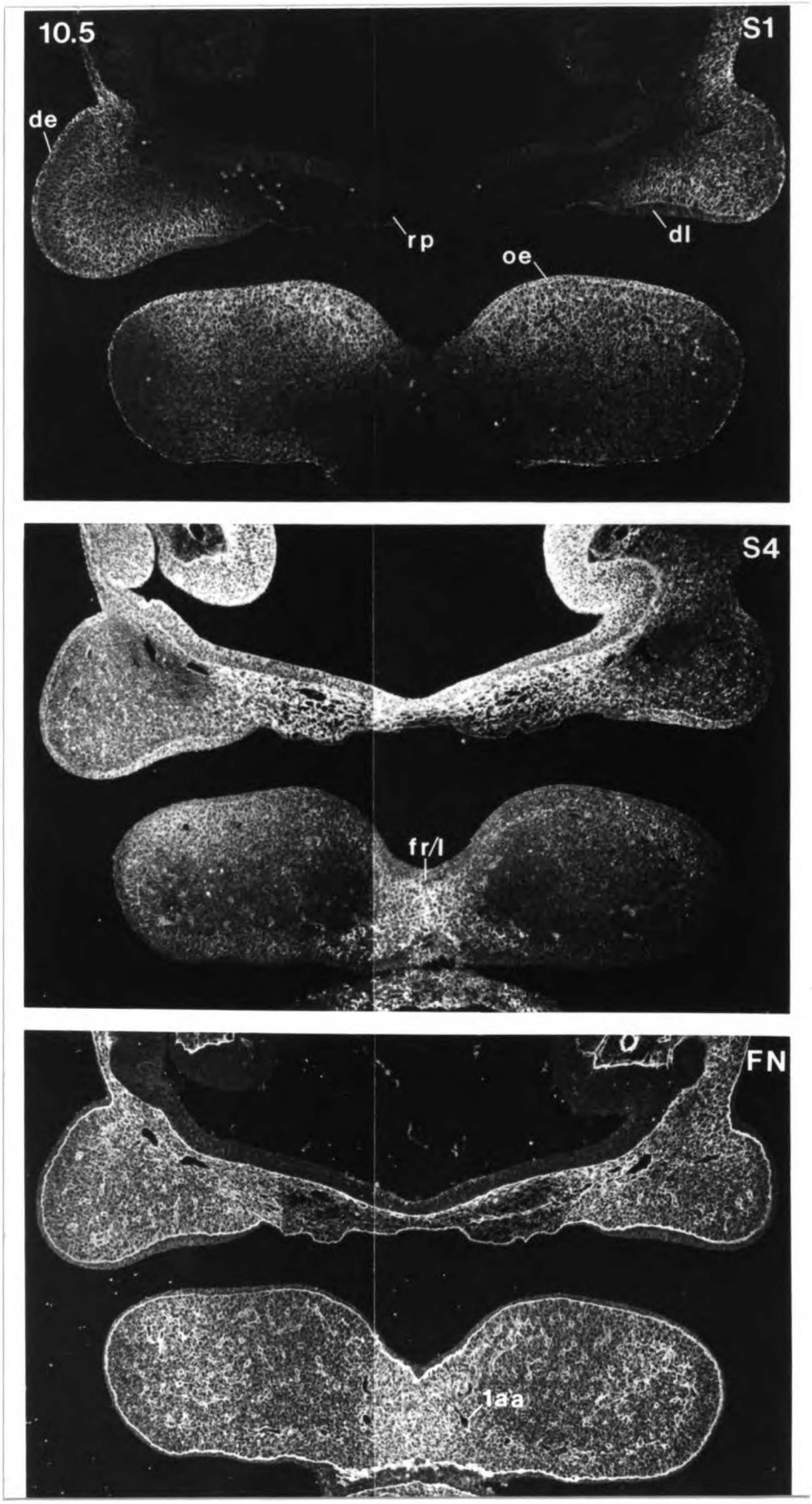
dl - dental lamina

f r/l - fusion right & left mandibular processes

oe - oral epithelium

rp - roof of pharynx

Plate 1b



11.5 days p.c. (Plates 2a-d)

Head - General distribution (Plate 2a)

S 1 Predominantly in first branchial arch, maxillary as well as mandibular component, and lateral to developing eye. Expression in dermal epithelium and narrow band of underlying mesenchyme. Mesenchymal expression weaker in posterior sections.

S 4 Predominantly in neuroepithelium of developing brain and in ganglia and nerves. Expression strongest on apical surface of epithelium and in underlying mesenchyme.

F N Expressed in mesenchymal tissues, strong expression in basement membranes.

Maxillary process & roof of pharynx

S1 Expression in dermal epithelium strongest on superior and inferior aspects of maxillary process, weak expression on lateral aspect of maxillary process. Mesenchymal expression on lateral & inferior aspect (Plates 2a,b). Expression very weak in developing dental lamina, with low expression in a narrow band of underlying mesenchyme. Mild epithelial expression in area where lateral palatal shelves are just beginning to develop, with mild expression in underlying mesenchyme as well (Plates 2b,c). Roof of pharynx shows a thin line of epithelial expression, no expression of S1 in underlying mesenchyme. A distinct line of S1 expression is present on the apical surface of the epithelium at the fusion of the maxillary and mandibular processes, which is an extension of the dermal expression of S1. Mesenchymal S1 expression at the line of fusion is limited to the dermal side (Plate 2c).

S 4 Very strong expression in nerve tissues. Dermal epithelium shows minimal apical expression, underlying mesenchyme on lateral side shows moderate band of expression (Plates 2a,b). The expression in the developing dental lamina in the

maxillary process has become more similar to the expression in the underlying mesenchyme. Oral epithelium overlying developing palatal shelf shows moderate expression, with a moderate band of expression in the underlying mesenchyme (Plates 2b,c). No S4 expression in mesenchyme of roof of pharynx.

F N Within maxillary process present throughout mesenchyme, most strongly in a vertical band which runs close to but not directly adjacent to the dermal epithelium on the lateral aspect (Plates 2a,b). Not expressed in nerve tissues.

Mandibular process

S 1 Epithelial expression is strongest in dermal epithelium and in anterior & lateral aspect of oral epithelium. Epithelial expression is very weak in developing dental lamina, with very low expression in underlying mesenchyme as well. Anteriorly (Plate 2b) a transverse band of moderate expression is located in the lower 1/3 of the mesenchyme, which extends to the lateral aspect of the mandibular process. There is reduced expression in the midline. More posteriorly (Plate 2c) mesenchymal expression is present in the pre-muscle mass of the lateral lingual swelling, in the lateral mesenchyme and in two circular pre-muscle mass areas adjacent to the midline. More posteriorly still (Plate 2d), two elliptical areas of slightly increased S1 expression are noted, at the primordium of Meckel's cartilage.

S 4 Strongly expressed in developing inferior alveolar and facial nerve. Mild apical expression in oral and dermal epithelium. Developing dental lamina shows minimal expression. Mild expression in mesenchyme underlying oral epithelium and inferior/ lateral dermal epithelium. In midline fusion area expressed in a narrow band of mesenchyme (Plates 2b,c). In the anterior area there is also mesenchymal expression in two circular areas directly adjacent to the midline (Plate 2b). This is where incisor formation is taking place. More posteriorly, a narrow band of

mesenchymal expression in lateral lingual swellings is seen (Plate 2c). At fusion area with maxillary process no epithelial S4 expression is noted, underlying mesenchyme shows mild S4 expression (Plate 2c). More posteriorly still, two ovoid areas of increased expression can be seen. This is presumed to be the pre-chondrogenic condensation of the posterior part of Meckel's cartilage (Plate 2d).

FN Within mandibular process present throughout mesenchyme. On anterior side (Plate 2b) expression is strongest in the lower 1/3, extending to the lateral aspect. Expression is weakest in the median aspect of the mandibular processes. More posteriorly (Plate 2c), a band of mild FN expression is noted in the pre-muscle mass of the lateral lingual swellings, as well as in two circular pre-muscle mass areas adjacent to the midline. Lateral to this structure a ring of increased expression is noted surrounding the developing inferior alveolar neurovascular bundle, with no FN expression present in the bundle itself. A band of mesenchymal FN expression runs parallel to but not directly adjacent to the dermal epithelium. More posteriorly still, two elliptical areas of slightly decreased FN expression are noted, at the primordium of Meckel's cartilage (Plate 2d).

Plate 2a. Frontal sections through head of 11.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. Morphogenetic movements continue to shape the maxillary and mandibular processes. Bar, 650 µm. ▬▬▬

dn - diencephalic neuroepithelium

eye - developing eye

mdp - mandibular process

mxp - maxillary process

tv - telencephalic vesicle

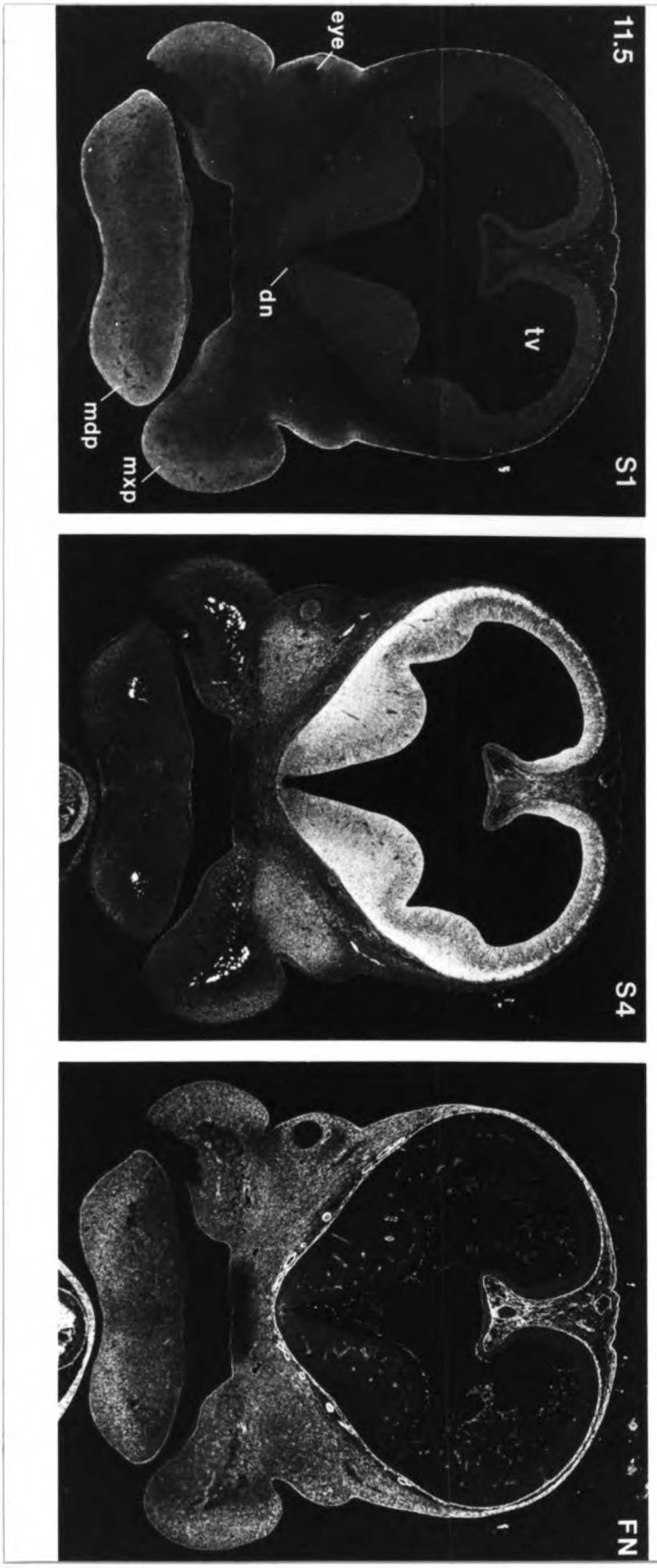


Plate 2a

Plate 2b. Same sections as plate 2a, close-up of maxillary and mandibular processes of 11.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. The first indication of the developing lateral palatal shelves is evident. Putative muscle primordia can be identified. The syndecan-4 stained section shows dental mesenchyme of the lower incisor, developing nerves, as well as the fusion line of the right and left mandibular processes. The defect in the maxillary process on the left side of the section is an artifact. Bar, 300 µm. ▬▬▬

de - dermal epithelium

dl - dental lamina

dm - dental mesenchyme

f r/l - fusion right & left mandibular processes

ian - inferior alveolar nerve

lps - lateral palatal shelf

nV - trigeminal nerve

oe - oral epithelium

pm - pre-muscle mass

Plate 2b

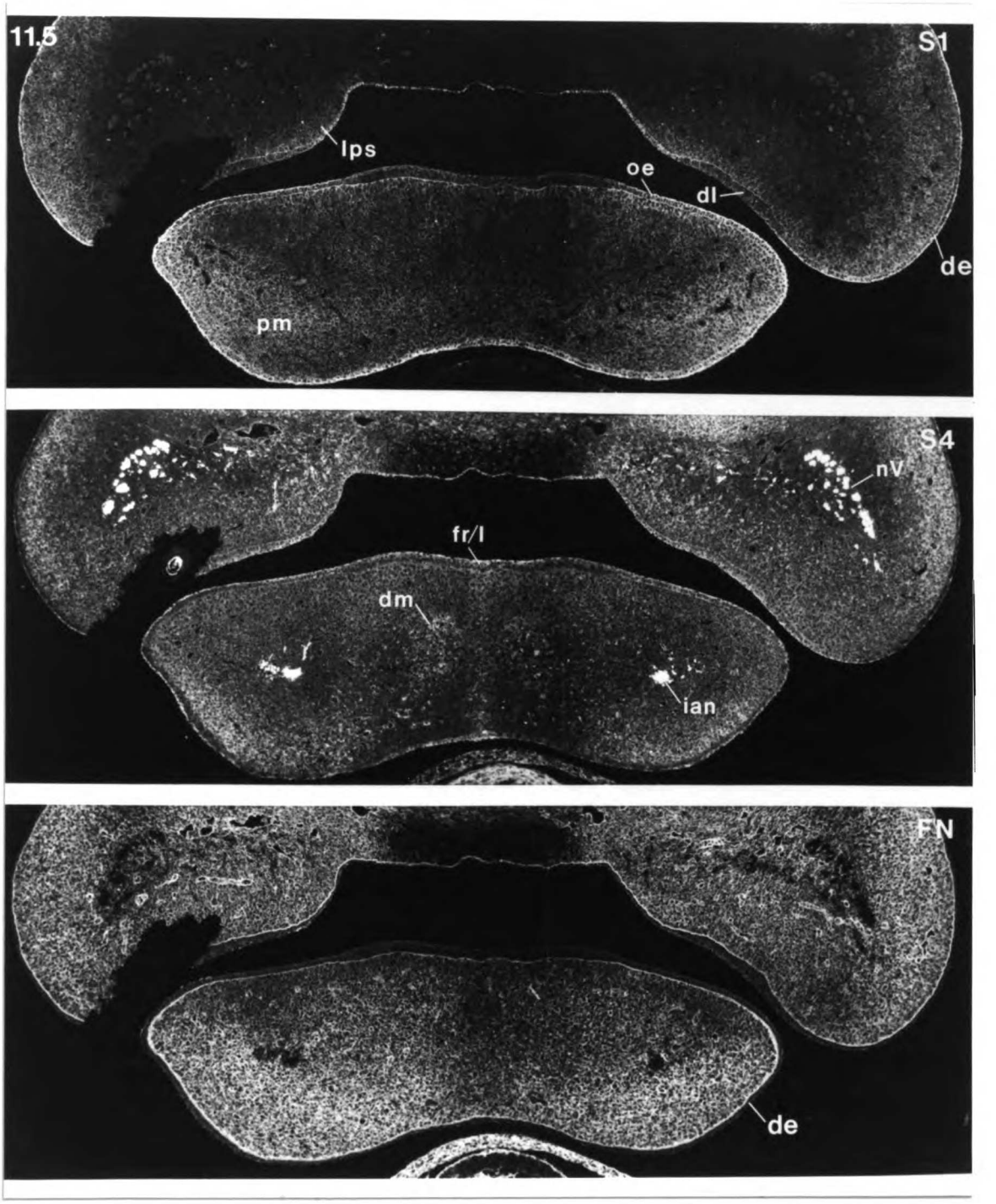


Plate 2c. Same specimen as plate 2a, deeper sections of maxillary and mandibular processes of 11.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. The lateral lingual swellings are evident, which will develop into the tongue. Bar, 300 μm. ▬▬▬▬

dl - dental lamina

f u/l - fusion maxillary & mandibular processes

ian - inferior alveolar nerve

lls - lateral lingual swelling

lps - lateral palatal shelf

lv - lingual vessels

nV - trigeminal nerve

pm - pre-muscle mass

Plate 2c

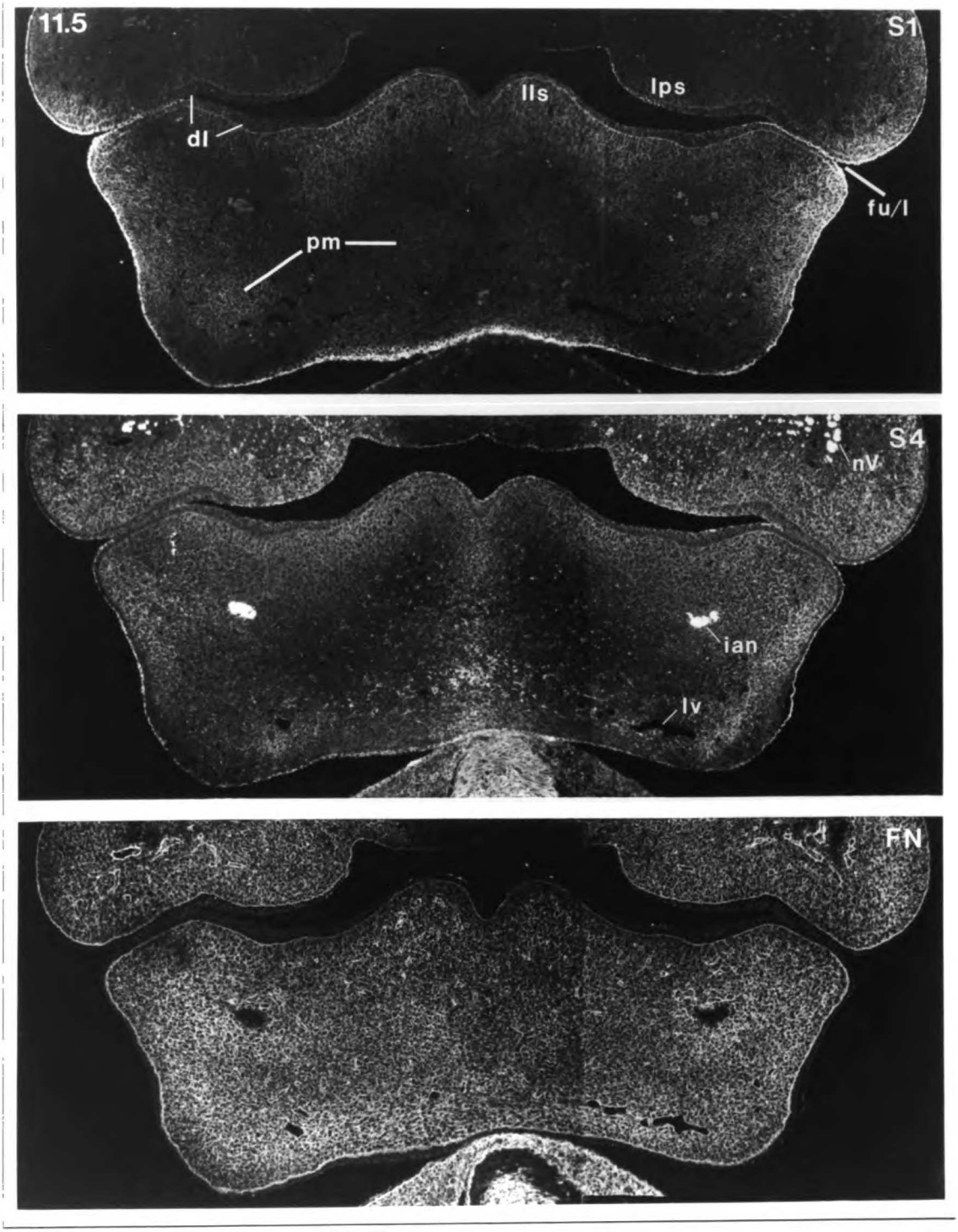


Plate 2d. Same specimen as plate 2a, deeper sections of maxillary and mandibular processes of 11.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. The first indication of Meckel's cartilage primordium can be identified, most easily in the section stained for syndecan-4. Bar, 300 μm. ▬▬▬▬

ian - inferior alveolar nerve

lls - lateral lingual swelling

ln - lingual nerve

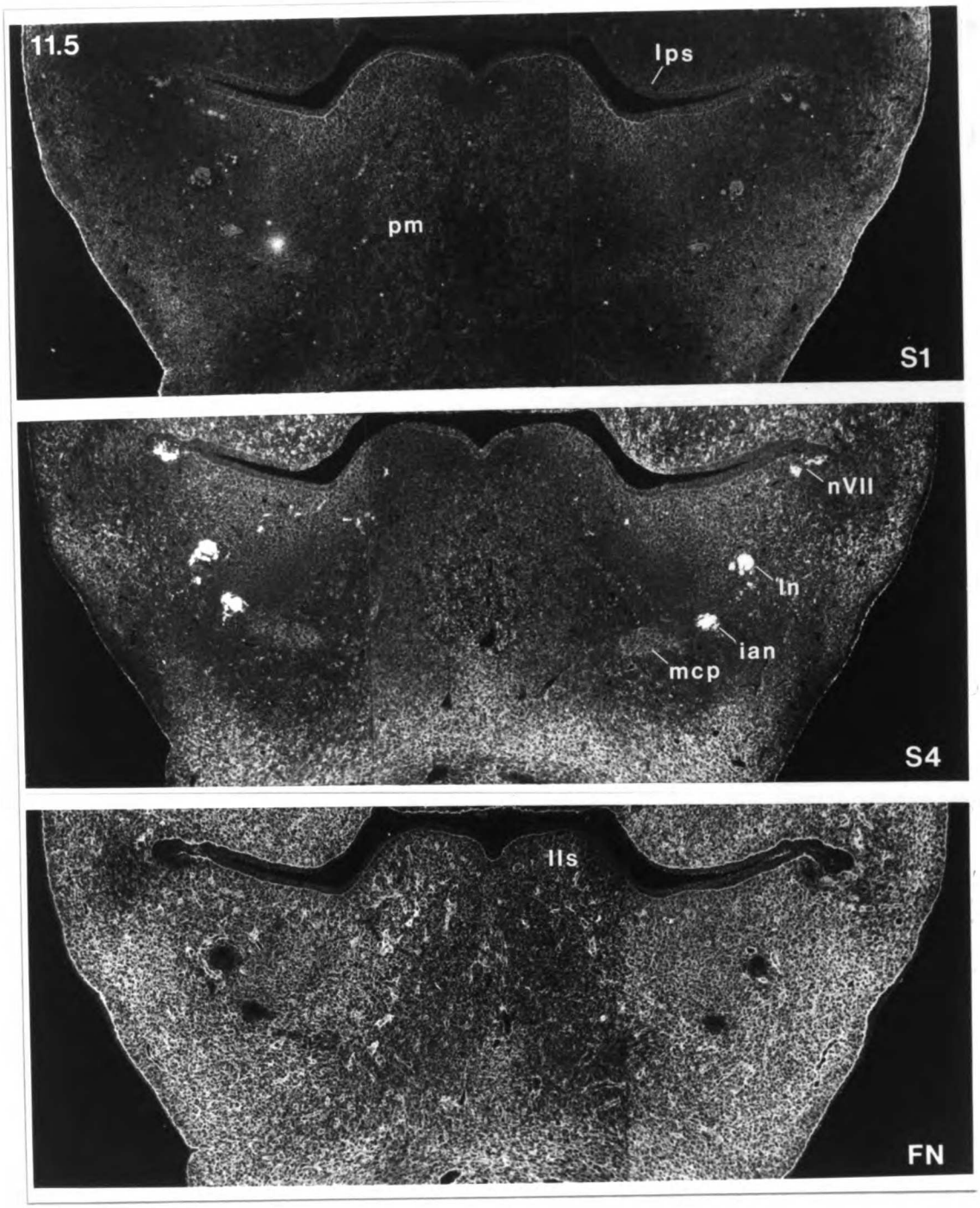
lps - lateral palatal shelf

mcp - Meckel's cartilage primordium

nVII - facial nerve

pm - pre-muscle mass

Plate 2d



12.5 days p.c. (Plates 3a-e)

Head - General distribution (Plate 3a)

S 1 Expression predominantly in dermal epithelium of lower half of head and narrow band of underlying mesenchyme.

S 4 Expression predominantly in neuroepithelium of developing brain and in ganglia and nerves and in muscle primordia.

F N Expressed in mesenchymal tissues, strong expression in basement membranes, in areas of future bone development such as the calvaria and the mandible and in association with muscle development.

Maxillary process & roof of pharynx

S 1 Expression in oral epithelium is low. Mild expression in lateral dermal epithelium, with no expression in underlying mesenchyme (Plate 3a). Strongest epithelial and mesenchymal expression on the inferior side, just lateral to where the maxillary process will fuse with the mandibular process. Epithelial expression is low at the future fusion area, with mild expression in underlying mesenchyme. A distinct line of S1 expression is present at the actual fusion of the maxillary and mandibular processes, which is an extension of the dermal expression of S1. Mesenchymal S1 expression at the line of fusion is limited to the dermal side (Plates 3b,c). Mild expression in epithelium of developing molar bud, with increased expression in surrounding mesenchyme (Plates 3c,d). Mild epithelial staining in lateral palatal shelves (Plate 3d).

S 4 Within maxillary process present in central and lower mesenchyme. Minimal expression in dermal epithelium and in epithelium on roof of nasal cavity (Plates 3a,b). Moderate expression on apical surface of epithelium of future fusion area between maxillary and mandibular processes with minimal expression in underlying

mesenchyme (Plate 3b). At the location where the epithelia of the maxillary and mandibular processes are in contact the apical expression of S4 is no longer present. Moderate apical epithelial expression at developing lateral palatal shelf (Plate 3c). Slightly increased expression in epithelium of developing molar bud, with decreased expression in surrounding mesenchyme (Plates 3c,d).

F N Within maxillary process strongest expression in a centrally located vertical band in association with muscle development (Plate 3a). Reduced expression in mesenchyme around dental lamina primordium of molar tooth bud (Plates 3c,d).

Mandibular process

S 1 Moderate expression in dermal epithelium and underlying mesenchyme. Expression in oral epithelium is low. Strongest mesenchymal S1 expression is seen at the lateral side underlying the area where fusion will take place between the maxillary and mandibular processes and underlying the adjacent dermal epithelium (Plates 3a,b). At the line of fusion there is a distinct line of apical epithelial expression while expression in the underlying mesenchyme is low (Plate 3c). Anteriorly minimal S1 staining is present in the dental lamina of the lower incisors with mild staining in the surrounding mesenchyme. Within the mandibular mesenchyme two elliptical areas of increased expression are noted adjacent to the midline (Plate 3b). More posteriorly this area extends more laterally (Plate 3c) until it forms a narrow band of enhanced S1 staining that traverses the full width of the mandibular processes (Plate 3d). This staining pattern seems to delineate the muscle primordia of the floor of the mouth. The inferior alveolar neurovascular bundle and surrounding mesenchyme show minimal to no S1 staining (Plates 3c,d). Two additional circular areas of increased expression are noted adjacent to the midline above the previously mentioned elliptical areas. This represents the formation of Meckel's cartilage (Plates 3c,d). Posteriorly, mesenchymal S1

staining is noted as a circular band around the developing Meckel's cartilage (Plate 3e). The first indication of oral and buccal vestibulum formation can be identified. These invaginating epithelial grooves show mild S1 staining (Plate 3d). S1 staining is very low in the area where mandibular bone will form at a later stage (Plate 3e).

S4 Epithelial S4 expression is noted throughout the epithelium on the dorsal surface of the tongue. It is also noted on the apical surface of the oral epithelium of the mandibular processes. There is minimal expression on the lateral borders of the tongue and on the dermal epithelium. The dental mesenchyme of the lower incisors can be identified as two circular areas of decreased S4 staining adjacent to the midline. The increased staining in two elliptical areas directly underneath could represent a muscle primordium. Expression of S4 is noted in the developing tongue musculature. A small round area of increased expression is seen in the midline just below the tongue. This is thought to be the developing Meckel's cartilage (Plate 3b). More posteriorly Meckel's cartilage primordium shows very minimal to no S4 expression (Plates 3c-e). There is a narrow band of mesenchymal S4 expression underlying the inferior dermal epithelium which extends laterally (Plates 3b-e). There is minimal S4 expression in the mesenchyme underlying the fusion area between the maxillary and mandibular processes (Plate 3c). The developing oral (Plates 3c-e) and buccal (Plates 3d,e) vestibules show strong mesenchymal S4 staining, with no epithelial S4 expression. The mesenchyme surrounding the inferior alveolar neurovascular bundle shows no S4 staining (Plates 3c-e).

FN Within the mandibular processes expressed most strongly in the lower border mesenchyme. Minimal expression in areas of incisor development, no expression in nerves. A small median circular area of expression is noted at the anterior side of the developing Meckel's cartilage (Plates 3a,b). Decreased FN stain is present in

more posterior areas of Meckel's cartilage formation (Plates 3c,d). More posterior still, a slight increase in FN stain of Meckel's cartilage is noted (Plate 3e). An increase of FN expression is noted in the area where ossification of the mandible will soon be initiated, lateral to the developing cartilage (Plates 3d,e). A reduction in FN expression is noted in the mesenchyme underlying the fusion area between the maxillary and mandibular prominence, with moderate expression in the mesenchyme underlying the adjacent dermal epithelium (Plate 3c).

Plate 3a. Frontal sections through head of 12.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. The tongue can now be identified, molar buds are beginning to develop. Bar, 760 μm. ▬▬▬▬

eye - developing eye

mb - molar bud

mdp - mandibular process

mxp - maxillary process

nV - trigeminal nerve

t - tongue

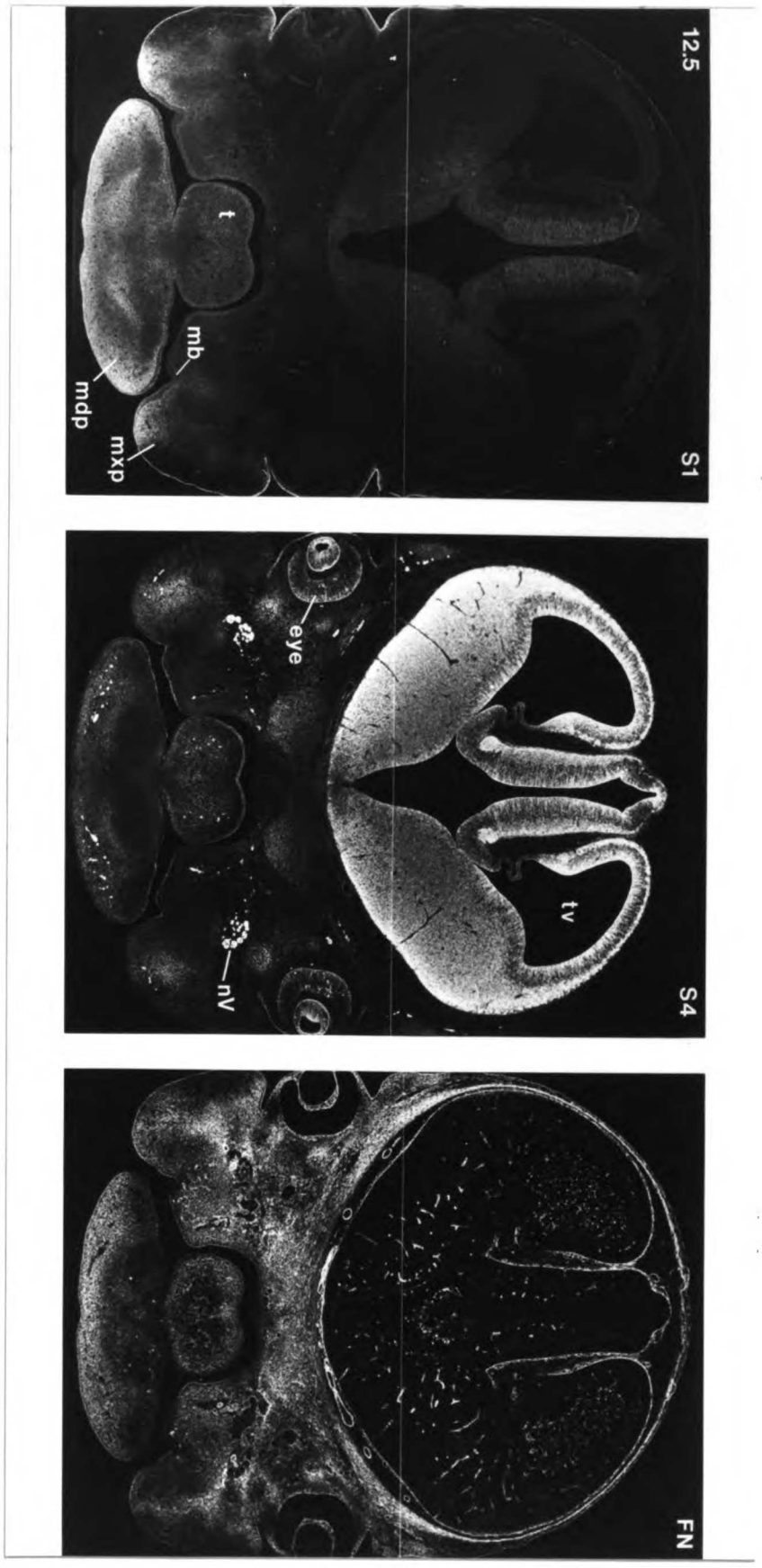


Plate 3a

Plate 3b. Same sections as plate 3a, close-up of maxillary and mandibular processes of 12.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. Further development of putative muscle primordia is evident. The dental lamina and dental mesenchyme of the lower incisors can be identified, as well as the developing molar buds in the maxillary processes. Bar, 400 μm. ▬▬▬▬

dl - dental lamina

dm - dental mesenchyme

lps - lateral palatal shelf

mb - molar bud

mn - mental nerve

mp - muscle primordium

nV - trigeminal nerve

Plate 3b

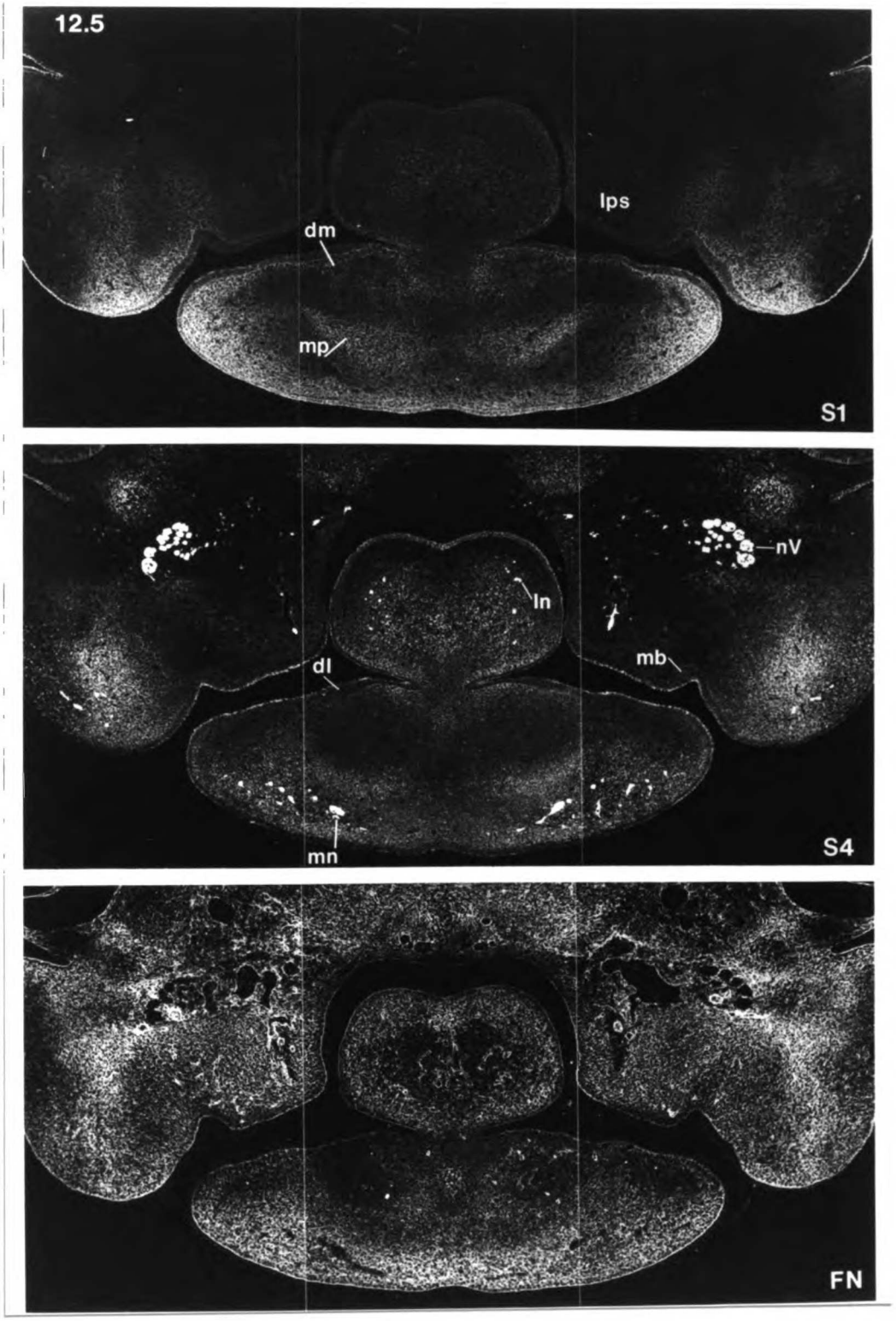


Plate 3c. Same specimen as plate 3a, deeper sections of maxillary and mandibular processes of 12.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. These sections give a more complete view of the maxillary molar buds. An epithelial thickening on the lateral aspect of the developing palatal shelves can be identified. Putative muscle primordia and directly above this Meckel's cartilage primordium can be seen in the mandibular component of the first branchial arch. The fusion area of the maxillary and mandibular processes can be identified. Bar, 400 μm. ▬▬▬▬

f u/l - fusion maxillary & mandibular processes

ian - inferior alveolar nerve

ln - lingual nerve

lps - lateral palatal shelf

mb - molar bud

mcp - Meckel's cartilage primordium

mn - mental nerve

nV - trigeminal nerve

pm - pre-muscle mass

Plate 3c

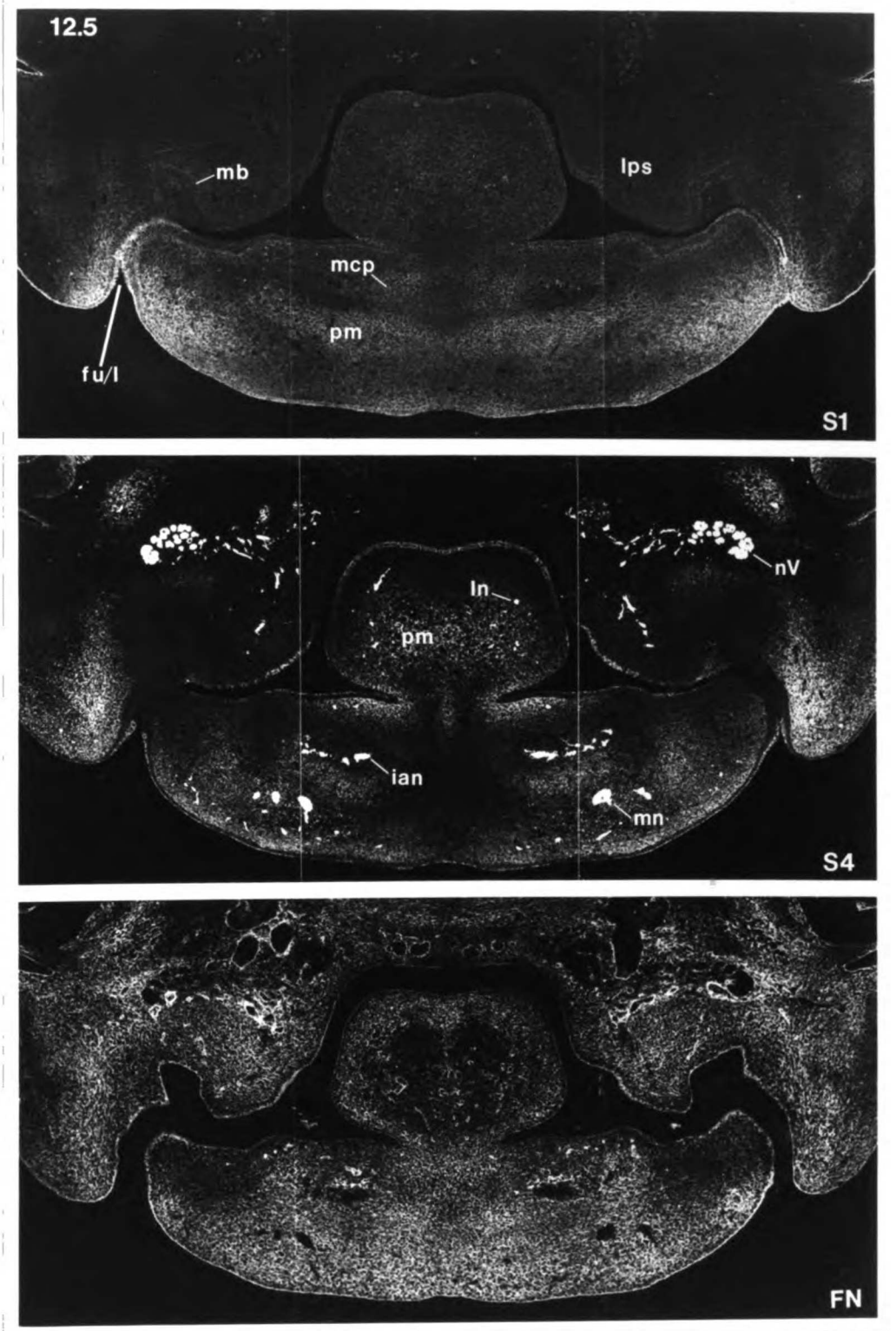


Plate 3d. Same specimen as plate 3a, deeper sections of maxillary and mandibular processes of 12.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. The first indication of buccal and oral vestibulum formation can be identified. Meckel's cartilage primordium is visible at either side of the midline.

Bar, 400 μm.

bv - buccal vestibulum formation

dm - dental mesenchyme

ian - inferior alveolar nerve

ln - lingual nerve

mb - molar bud

mcp - Meckel's cartilage primordium

nV - trigeminal nerve

nVII - facial nerve

ov - oral vestibulum formation

pm - pre-muscle mass

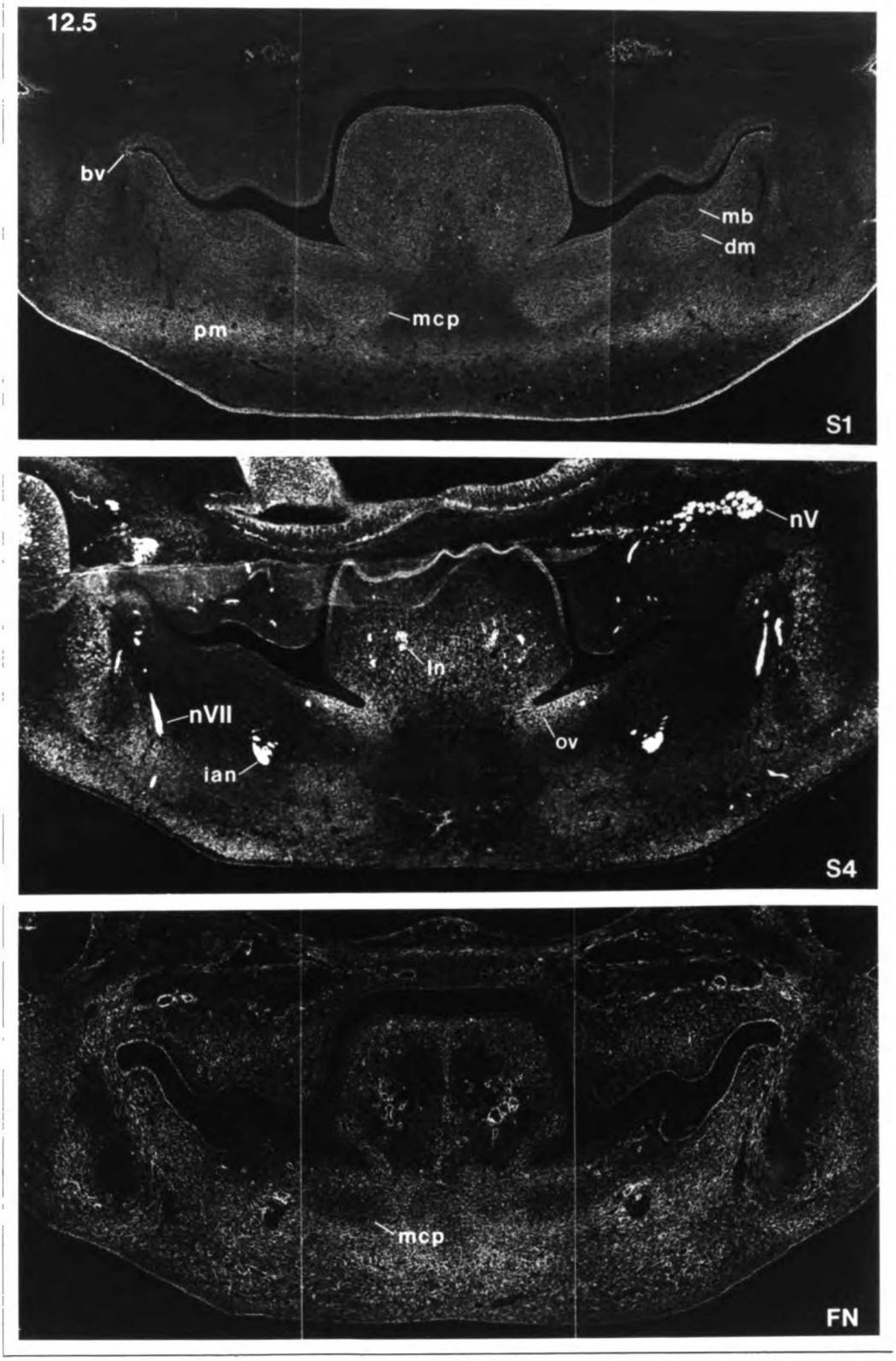

12.5
bv
mb
dm
pm
mcp
S1
nV
ln
nVII
ian
ov
S4
mcp
FN

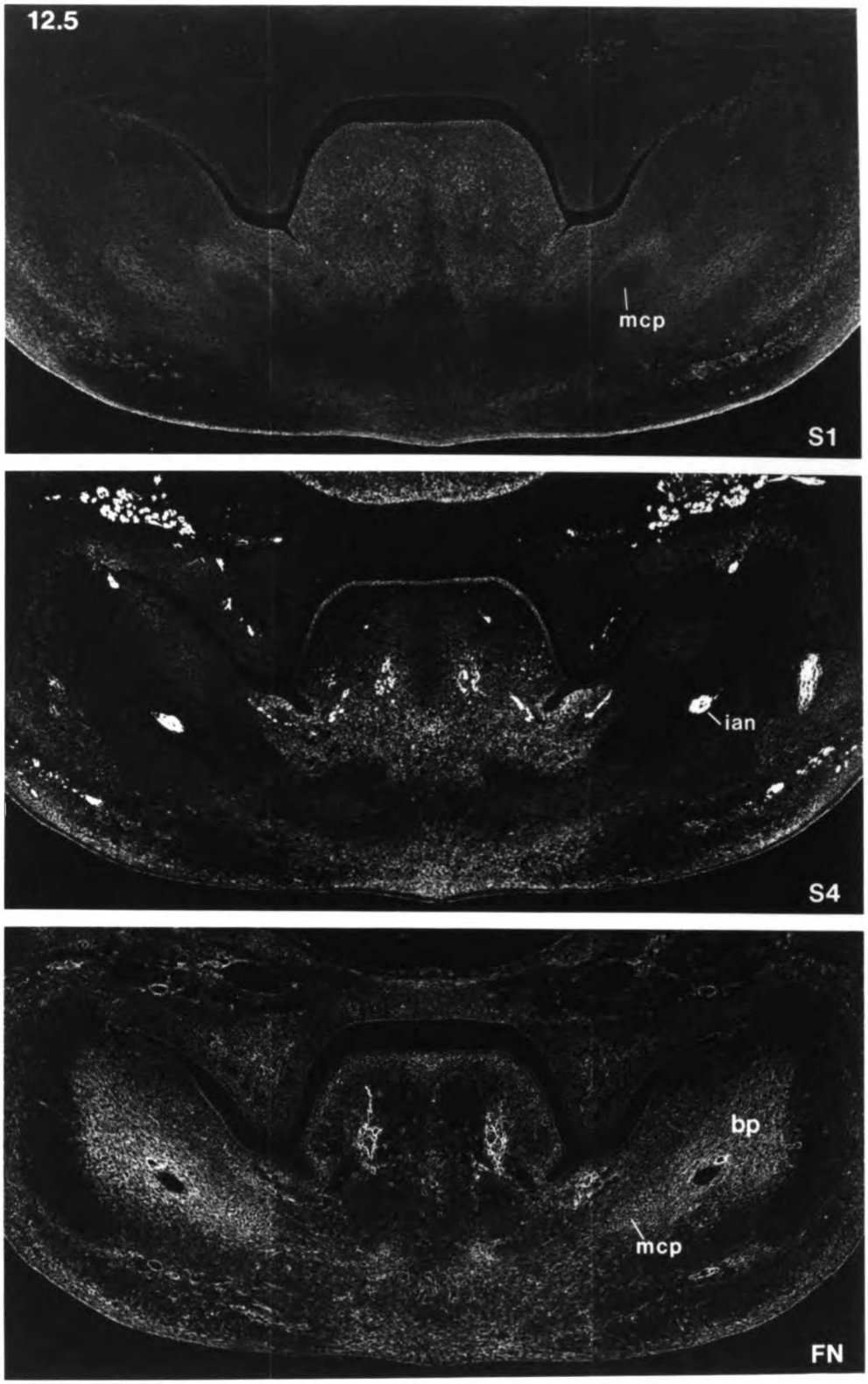
Plate 3e. Same specimen as plate 3a, deeper sections of maxillary and mandibular processes of 12.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. A more mature section of Meckel's cartilage primordium is visible at greater distance from the midline. The area of future mandibular bone formation can be identified in the section stained for fibronectin. Bar, 400 μm. ▬▬▬▬

mcp - Meckel's cartilage primordium

ian - inferior alveolar nerve

bp - bone primordium

Plate 3e



13.5 days p.c. (Plates 4a,b and 5a-c)

Head - General distribution (Plate 5a)

S 1 Expression in dermal epithelium of lower half of head with very little expression in underlying mesenchyme.

S 4 Expression predominantly in neuroepithelium of developing brain and in ganglia and nerves and in muscle primordia.

F N Expressed in mesenchymal tissues, strong expression in basement membranes, in areas of bone development such as the calvaria and the mandible and in areas of muscle development. Expression in wall of arteries noted.

Maxillary process & roof of pharynx

S 1 Minimal expression in epithelium in future fusion area with moderate expression in underlying mesenchyme. Moderate expression in adjacent dermal epithelium . The epithelium of the developing lateral palatal shelves shows strong S1 expression (Plate 4a). Moderate expression in epithelium of developing molar bud, with strong expression in surrounding mesenchyme (Plate 4a). Expressed in nasal septum cartilage (Plate 5a).

S 4 Minimal expression in epithelium or mesenchyme in fusion area with mandibular process. Expressed in mesenchyme adjacent to fusion area with mandibular process (Plate 4a). Distinct apical line of expression in dental lamina (Plates 4a, 5b,c), and in core of dental lamina (Plate 5c) with minimal expression in epithelium of developing molar bud, or in surrounding mesenchyme (Plates 4a, 5b,c). Sharply increased expression on apical surface of epithelial thickening on lateral part of developing lateral palatal shelf. Minimal expression in underlying mesenchyme (Plates 4a, 5a,b). The medial mesenchyme of the developing lateral palatal shelves shows a mild band of increased S4 expression (Plate 5a,b).

FN Minimal expression in mesenchyme around dental lamina primordium of molar tooth bud (Plate 5c) . Expressed in outer part of nasal septum cartilage (Plate 4b, 5a). Moderate mesenchymal expression underlying dermal epithelium and underlying fusion area with mandibular process (not shown).

Mandibular process

S1 Strong expression in dermal epithelium and narrow band of underlying mesenchyme. Minimal expression in oral epithelium, moderate expression on dorsal & lateral surfaces of tongue (Plates 4a, 5a,b). Minimal epithelial expression in fusion area with maxillary process, stronger expression in adjacent dermal epithelium. Strong staining of the mesenchyme at the lateral border in fusion area. Strong staining in the mesenchyme around the incisor enamel organs (Plate 4a), strong expression in the mesenchyme around the molar buds (Plate 5c). Very low expression in areas of mandibular bone formation. In posterior sections the mesenchymal expression of S1 is very faint (Platse 5a-c). Meckel's cartilage is surrounded by a band of S1 positive mesenchyme (Plates 5a-c). The developing oral vestibulum epithelium shows strong S1 staining. The developing buccal vestibulum epithelium also shows increased S1 expression (Plates 5a-c).

S4 Epithelial S4 expression is noted throughout epithelium on dorsal and lateral surfaces of tongue. It is also noted at apical surface of dermal epithelium and in underlying mesenchyme, on apical surface of oral epithelium and on apical surface of epithelium in future fusion area between maxillary and mandibular prominence. After these epithelia come into contact this apical line of S4 expression disappears (Plate 4a). Thin, distinct apical line of expression in epithelium at site of molar bud invagination. Very mild expression around and in core of developing molar buds (Plate 4a, 5b,c). Present in enamel organ and surrounding mesenchyme of developing incisors (Plate 4a). A faint band of staining is seen surrounding

Meckel's cartilage (Plate 5b). Mild expression in areas of mandibular bone formation. Expression in several muscle primordia, such as m. genioglossus (Plate 5c). The developing oral vestibulum shows low epithelial S4 expression, with increased S4 expression in the surrounding mesenchyme. The developing buccal vestibulum epithelium also shows decreased S4 expression, with increased S4 expression in surrounding mesenchyme. Reciprocity with S1 expression is noted in this area (Plates 5a-c).

F N Expressed in wall of inferior alveolar vessels (Plate 4b, 5b,c). Expressed in areas of mandibular bone formation. Expressed in outer layer of Meckel's cartilage and immediate surrounding mesenchyme (Plate 4b, 5b,c). Expressed in muscle primordia, for example m. genioglossus (Plate 5b). Mesenchyme around oral and buccal vestibulum epithelium shows increased FN expression (Plate 5a-c).

Plate 4a. Frontal sections through maxillary and mandibular processes of 13.5 days p.c. mouse embryo, stained for syndecan-1 and syndecan-4. Bell stage of lower incisor development is evident in the mandible. Meckel's cartilage is present in between the developing lower incisors. Molar buds can be seen in the maxilla. Continued morphological development of the lateral palatal shelves is evident, they are still vertically directed adjacent to the tongue. Bar, 400 µm. ▬▬▬▬

dm - dental mesenchyme

eo - enamel organ

f u/l - fusion maxillary & mandibular processes

lps - lateral palatal shelf

mb - molar bud

mc - Meckel's cartilage

Plate 4a

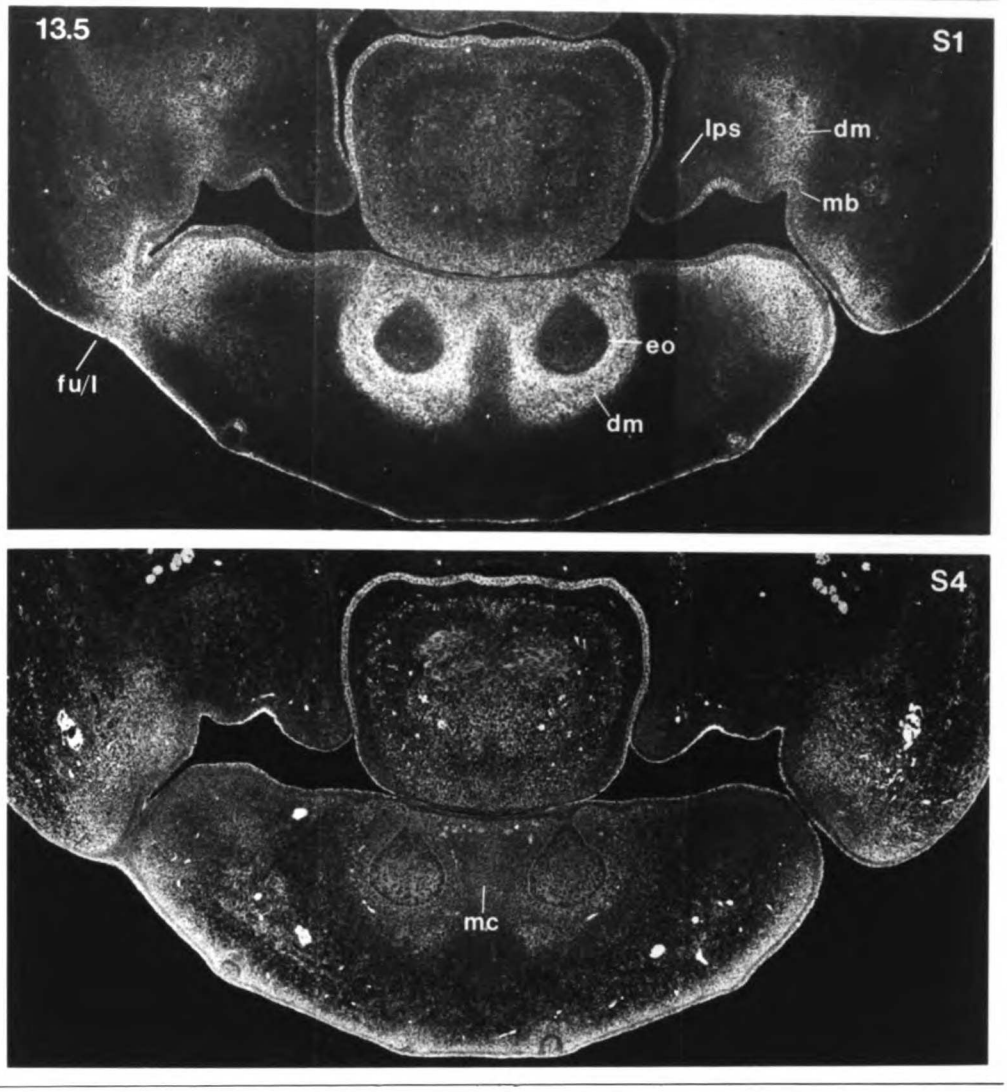


Plate 4b. Same specimen as plate 4a, deeper section through head of 13.5 days p.c. mouse embryo, stained for fibronectin. Bone formation of the calvaria as well as initial bone formation of the mandible is evident. The nasal septum and Meckel's cartilage can be identified. Bar, 410 µm. ▬▬▬▬

bfc - bone formation calvaria

bfm - bone formation mandible

iav - inferior alveolar vessels

llv - left lateral ventricle

mc - Meckel's cartilage

ns - nasal septum

t - tongue

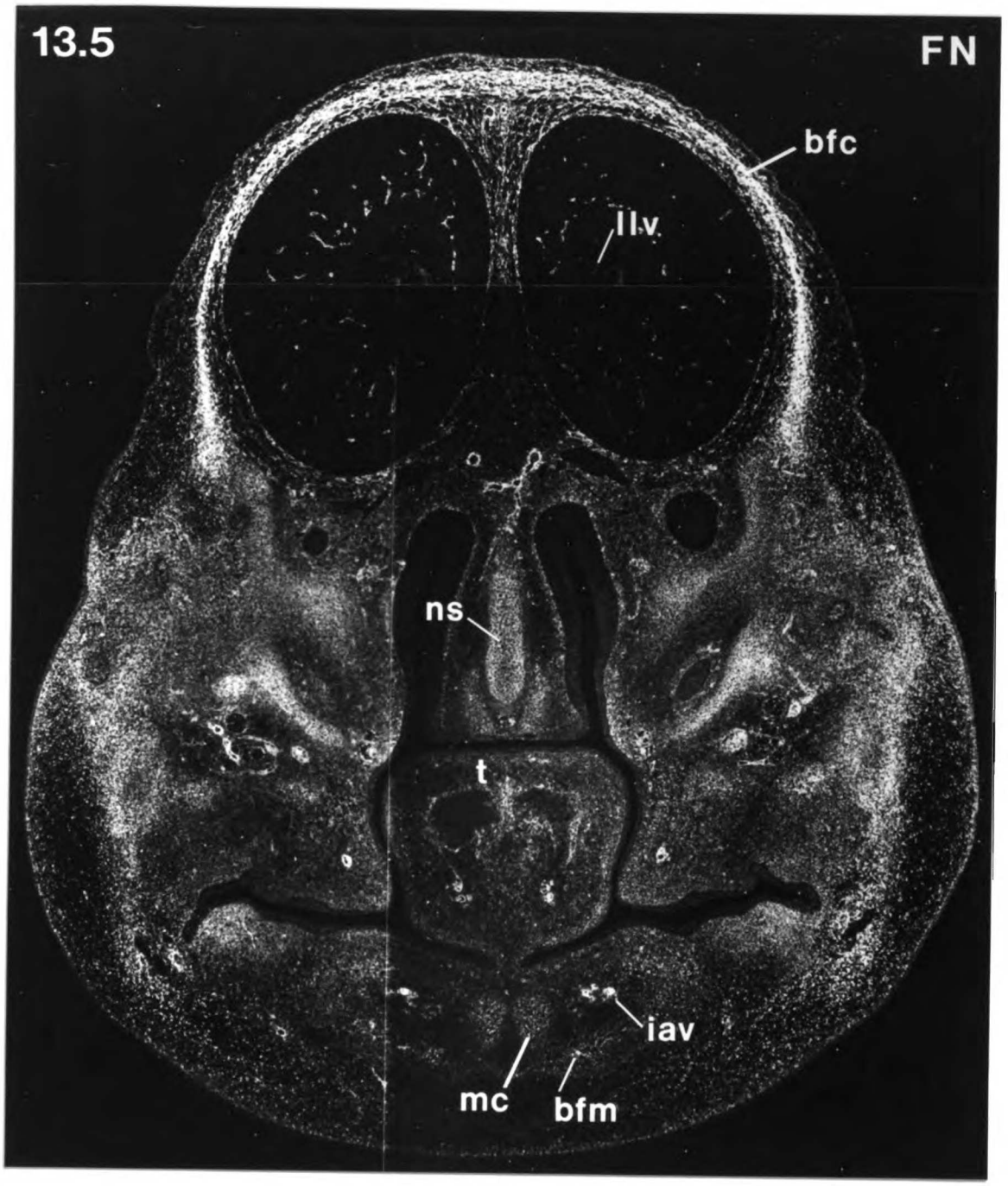

13.5
FN
bfc
llv
ns
t
iav
mc
bfm

Plate 5a. Frontal sections through head of 13.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. More posterior sections as compared to previous 13.5 days p.c. specimen (figures 12 & 13). Meckel's cartilage and the nasal septum can be identified. Bar, 750 μm. ▬▬▬

lps - lateral palatal shelf

mc - Meckel's cartilage

ns - nasal septum

t - tongue

Plate 5a

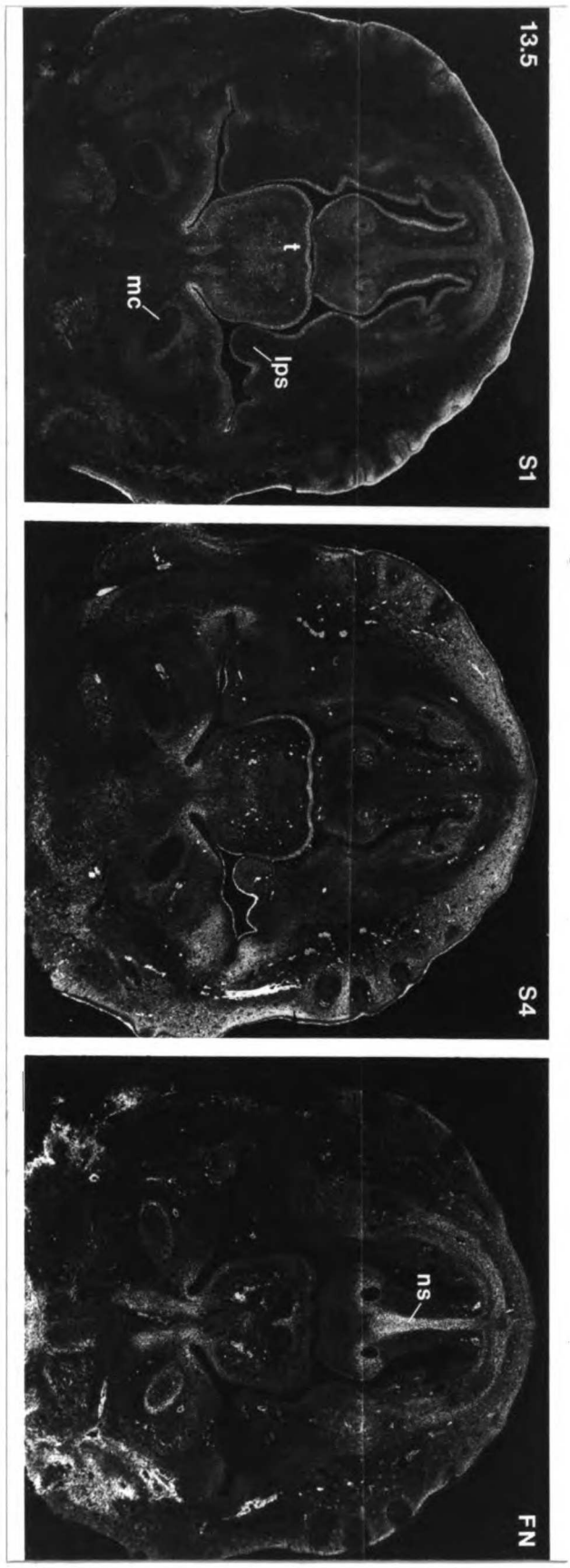


Plate 5b. Same sections as plate 5a, close-up of maxillary and mandibular processes of 13.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. These sections were made at a slight slant, so that the right side of the specimen represents a more anterior area than the left side. The more advanced stage of development of the lateral palatal shelf in the anterior area (right side of each image) as compared to the posterior area (left side of each image) can be clearly seen. The elevation of the palatal shelves starts in the anterior area and progresses posteriorly. Further development of the oral and buccal vestibulum is evident. On the superior/lateral aspect of Meckel's cartilage the inferior alveolar neurovascular bundle can be identified. Bar, 400 μm. ▬▬▬

bv - buccal vestibulum formation

ian - inferior alveolar nerve

lps - lateral palatal shelf

mb - molar bud

mc - Meckel's cartilage

mg - musculus genioglossus

nVII - facial nerve

ov - oral vestibulum formation

Plate 5b

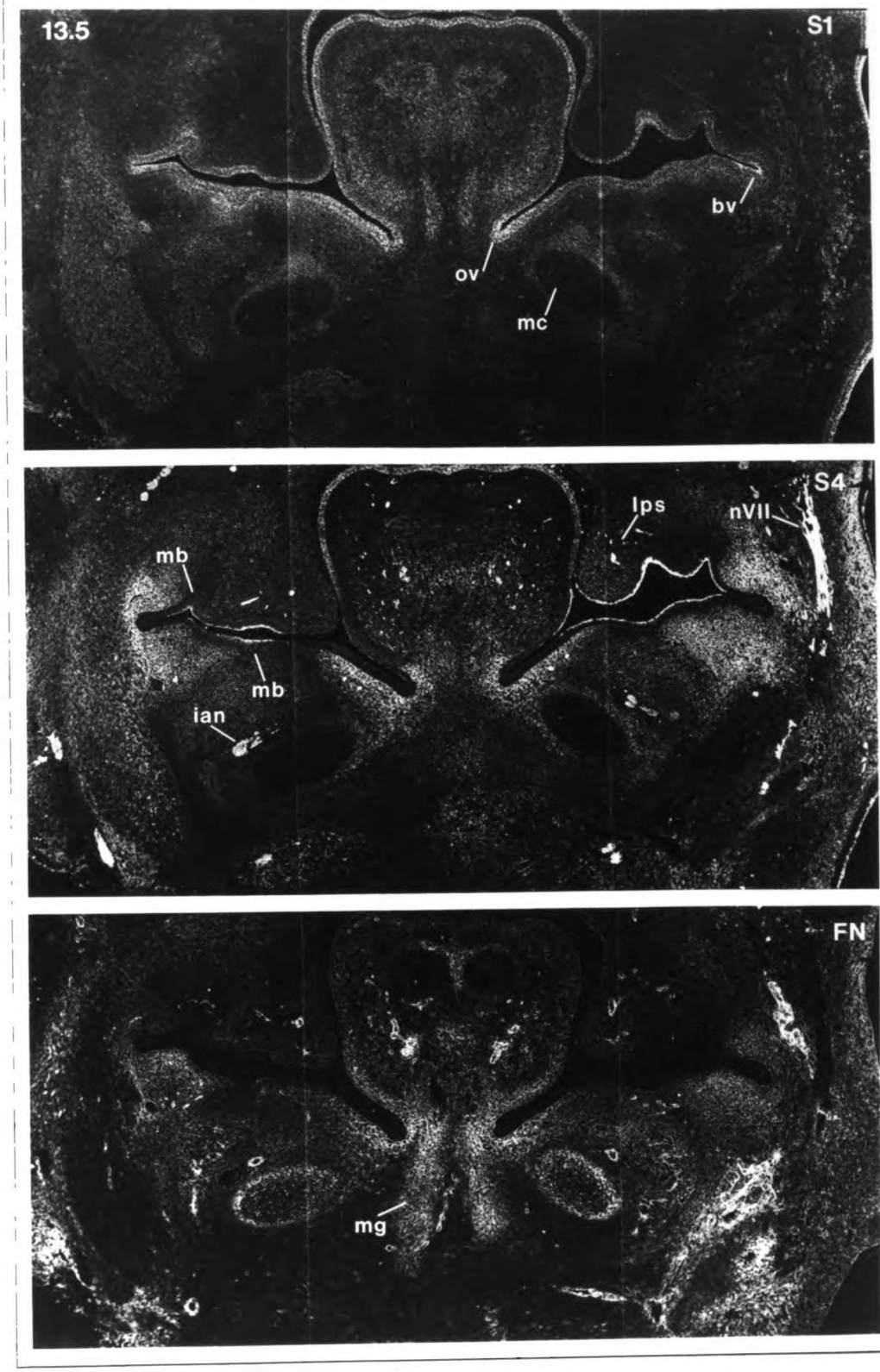


Plate 5c. Same specimen as plate 5a, deeper sections of maxillary and mandibular processes of 13.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. Progression of molar bud development is evident. The right submandibular gland and m. genioglossus can be identified. Mandibular bone formation is taking place lateral to Meckel's cartilage. Bar, 400 μm. ▬▬▬▬

bv - buccal vestibulum formation

ian - inferior alveolar nerve

lps - lateral palatal shelf

mb - molar bud

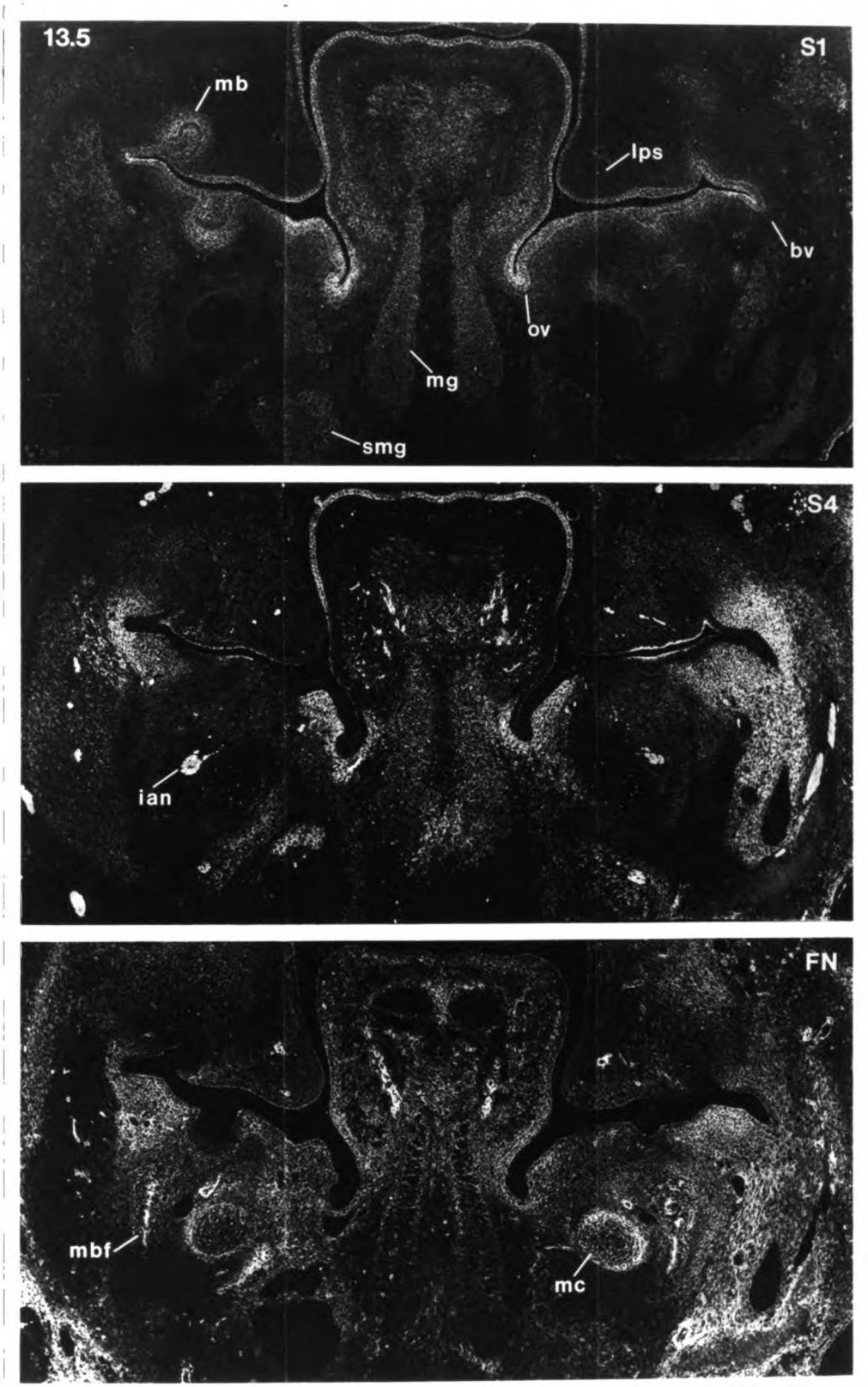
mbf - mandibular bone formation

mc - Meckel's cartilage

mg - musculus genioglossus

ov - oral vestibulum formation

smg - submandibular gland


13.5
S1
mb
lps
bv
ov
mg
smg
S4
ian
FN
mbf
mc

14.5 days p.c. (Plates 6a-c)

Head - General distribution (Plate 6a)

S 1 Expression predominantly in dermal epithelium, mild expression in oral and nasal epithelium.

S 4 Expression predominantly in neuroepithelium of developing brain and in ganglia and nerves and in muscle primordia.

F N Expressed in mesenchymal tissues, strong expression in basement membranes, in areas of bone development such as the calvaria and the mandible and in areas of muscle development. Expression in wall of arteries noted.

Maxillary process & roof of pharynx

S 1 Expressed in oral epithelium and dermal epithelium. In the core of the molar bud epithelium and in the mesenchyme directly around the developing molar bud S1 staining is evident (Plate 6c). Little expression in epithelium of future fusion area between maxillary and mandibular processes. Little expression in underlying mesenchyme (Plate 6b). Moderate expression on epithelium of elevated palatal shelves. (Plates 6b,c).

S 4 Expression on apical surface of dermal epithelium and underlying mesenchyme. Mild line of expression in center of developing molar bud, minimal expression in surrounding mesenchyme (Plate 6c). Mild expression on apical surface of epithelium of fusion area between maxillary and mandibular processes. Mesenchymal expression at fusion area limited to dermal and oral corner of mesenchyme (Plate 6b). Moderate expression on apical surface of oral epithelium. Moderate expression on epithelium of elevated palatal shelves (Plates 6b,c).

F N Expressed in mesenchyme underlying dermal epithelium. Minimal expression in mesenchyme around molar tooth bud (Plate 6c). Mild expression in mesenchyme

underlying fusion area of maxillary and mandibular processes. Strong expression on outer edge of cartilaginous nasal septum (Plate 6a).

Mandibular process (Plates 6a-c)

S 1 Strong expression in dermal epithelium, moderate expression in oral epithelium. Little expression in underlying mesenchyme. Little expression in epithelium or mesenchyme of future fusion area between maxillary and mandibular processes (Plate 6b). Strong expression in mesenchyme around developing incisors, mild expression in epithelial incisor cap. Butterfly shaped border of mild expression around bone and cartilage forming area. Low expression in bone forming areas, no expression in cartilage forming areas. The epithelium of the developing oral and buccal vestibules show persistent S1 expression, with no S1 expression in the surrounding mesenchyme. Mild expression in areas of muscle development.

S 4 Expression on apical surface of dermal epithelium and apical surface of oral epithelium and underlying mesenchyme. Expressed throughout dorsal and lateral tongue epithelium. Expressed on apical surface of epithelium on lateral side of fusion line between maxillary and mandibular processes, in continuation with dermal expression. Reduced apical epithelial expression in the center of the fusion line. Expressed in median corners of mesenchyme in maxillary/ mandibular fusion area, no S4 expression in narrow band of mesenchyme directly underlying epithelium at fusion site. After fusion expression in mesenchyme bridging maxilla and mandible on dermal side and on oral side. Expressed around developing incisors and in core of incisor cap epithelium (Plates 6a,b). Mild expression in bone forming areas of mandible, for example directly below Meckel's cartilage, but not present in newly formed bone spicules (Plate 6c). No expression in cartilage. Expression in areas of muscle development, for example in the tongue. Little expression in epithelium of developing oral and buccal vestibules, with strong

expression in the surrounding mesenchyme. Again S1 and S4 expression is reciprocal in the epithelium and mesenchyme in this area (Plates 6b,c).

FN Highly expressed in bone forming areas, especially in newly formed bone spicules. Expressed in mesenchyme underlying dermal epithelium. Expressed in mesenchyme underlying fusion area of maxillary and mandibular processes. After fusion expression in mesenchyme bridging maxilla and mandible on dermal side and on oral side (Plate 6b). Mild expression around developing incisors. Strong expression in anterior midline between right and left halves of developing mandible (Plate 6b). FN expression in the tongue is reciprocal with S4 and S1 expression. Expressed in outer layer of nasal septum cartilage. Expressed in outer layer of Meckel's cartilage and immediate surrounding mesenchyme. Increased expression in mesenchyme around developing oral and buccal vestibulum (Plates 6a-c).

Plate 6a. Frontal sections through head of 14.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. The palatal shelves have elevated above the tongue. They are apparently not touching in the midline, however this is presumed to be a processing artifact (Diewert and Tait, 1979). Bar, 875 µm. ▬▬▬▬

dm - dental mesenchyme
eo - enamel organ
eye - developing eye
jo - Jacobson's organ
mc - Meckel's cartilage
ne - neuroepithelium
ns - nasal septum
rlv - right lateral ventricle
t - tongue

Plate 6a

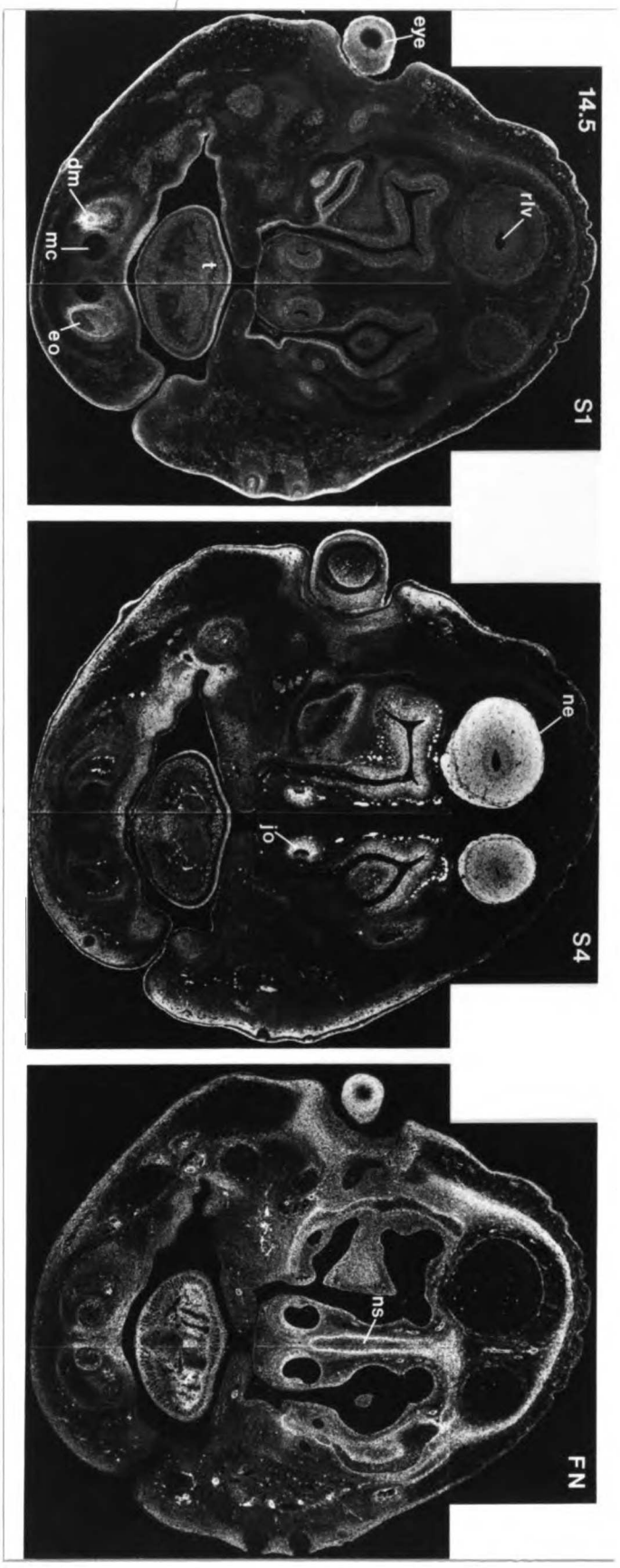


Plate 6b. Same sections as plate 6a, close-up of maxillary and mandibular processes of 14.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. An intricate pattern of muscle orientation can be seen in the tongue. In the mandible further incisor development is taking place. Meckel's cartilage can be seen close to the midline. Bar, 400 μm. ▬▬▬▬

dm - dental mesenchyme

eo - enamel organ

ian - inferior alveolar nerve

ltm - longitudinal tongue musculature

mc - Meckel's cartilage

mf - muscle fasciae

pv - palatine vessels

ttm - transverse tongue musculature

Plate 6b

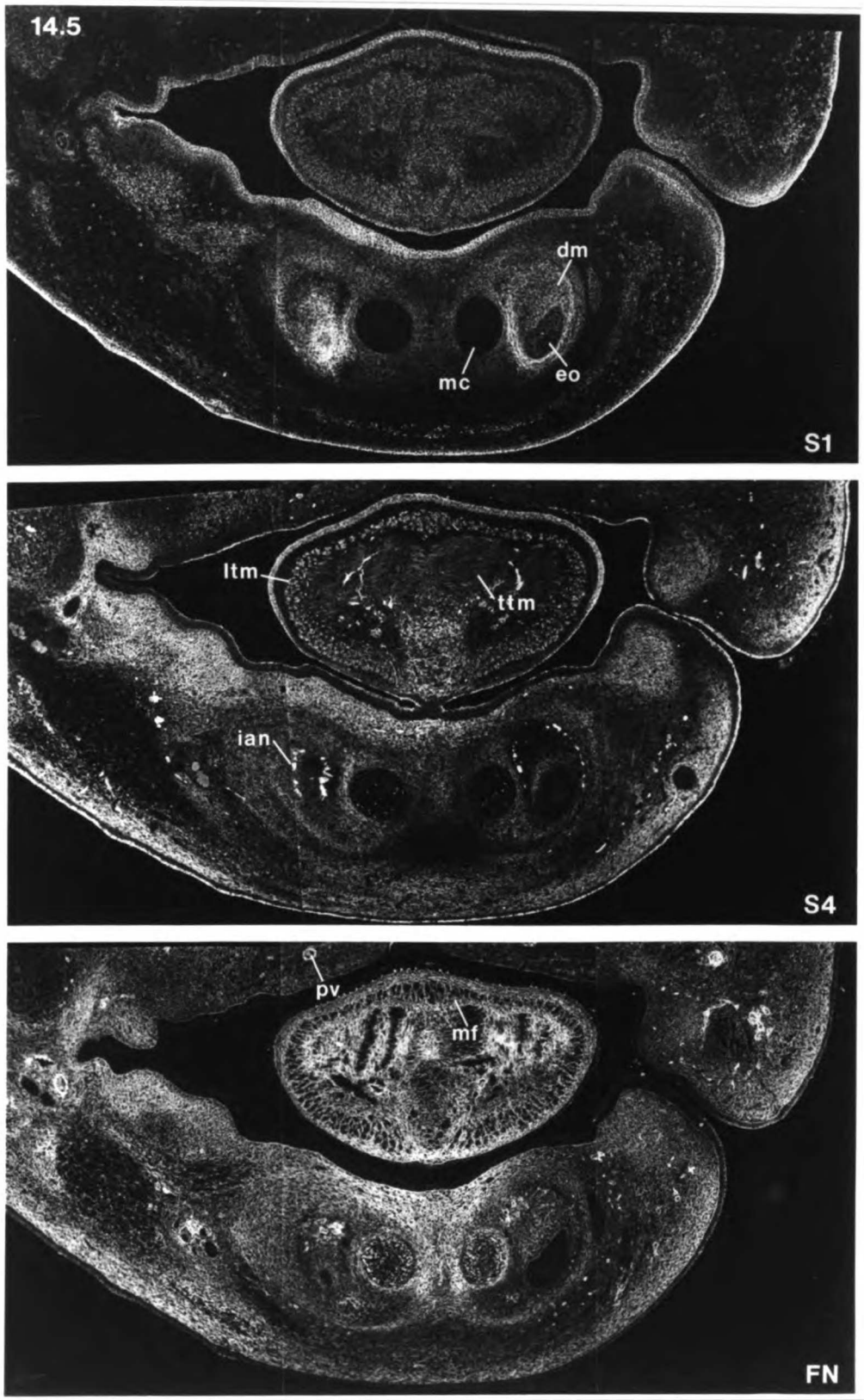


Plate 6c. Same specimen as plate 6a, deeper sections of maxillary and mandibular processes of 14.5 days p.c. mouse embryo, stained for syndecan-1, syndecan-4 and fibronectin. Extensive bone formation is taking place in the mandible, especially on the more posterior side (left side of each image). Bar, 400 μm. ▬▬▬

bv - buccal vestibulum formation

eps - elevated palatal shelf

ian - inferior alveolar nerve

mb - molar bud

mbf - mandibular bone formation

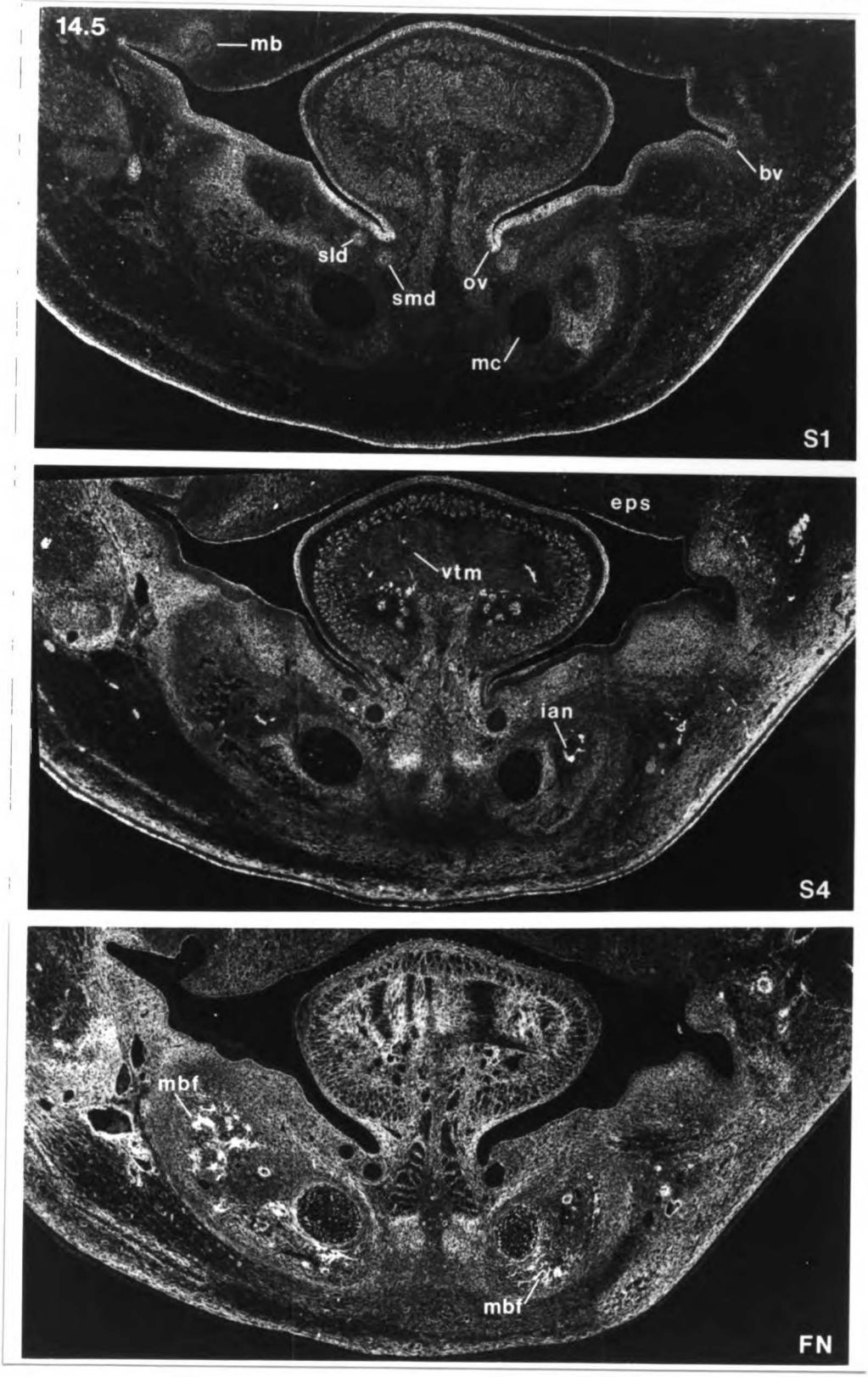
mc - Meckel's cartilage

ov - oral vestibulum formation

sld - sublingual salivary duct

smd - submandibular salivary duct

vtm - vertical tongue musculature


14.5
mb
bv
sld
smd
ov
mc
S1
eps
vtm
ian
S4
mbf
mbf
FN

Discussion

Syndecan-1, syndecan-4 and fibronectin show distinct expression patterns in relation to specific developmental processes in the mouse head. The correlations between these expression patterns and several morphogenetic and differentiation events that take place during the period studied (10.5 to 14.5 days p.c.) are outlined below.

Central fusion of right and left mandibular processes

At 10.5 days p.c. the right and left mandibular processes are in the process of fusing in the midline. At this point both syndecan-4 and fibronectin are expressed strongly in the midline mesenchyme of the mandibular process. In addition syndecan-4 shows slightly increased epithelial expression in the midline. In contrast, syndecan-1 expression is low in the midline mesenchyme and epithelium. As development continues to 11.5 days p.c. syndecan-4 expression remains strong in a narrow band of midline mesenchyme. Fibronectin expression in the midline mesenchyme has decreased. Syndecan-1 expression remains weak in the midline mesenchyme region, but expression across the dermal epithelium has become continuous.

This pattern of expression suggests that syndecan-1 , syndecan-4 and fibronectin play a functional role in the events that lead to the fusion of the right and left mandibular processes. The absence of syndecan-1 , and the presence of syndecan-4 and fibronectin could be a prerequisite for the epithelial and mesenchymal fusion to be completed. The decreasing expressions of syndecan-4 and fibronectin and the appearance of syndecan-1 in the midline area during later stages after the fusion has been completed suggest a functional link. Syndecan-4 and fibronectin are present during the active fusion stage and disappear after the process has been completed. Syndecan-1 is absent during the active fusion stage

and appears after the process has been completed. Loss of epithelial syndecan-1 expression has been documented during another fusion event in craniofacial development, the fusion of the elevated palatal shelves. Epithelial cells in the midline seam loose staining for syndecan-1 , while mesenchymal staining becomes increased (Brinkley et al., 1992). Further studies are needed to describe the expression patterns of syndecan-1 and syndecan-4 during the earlier stages of the fusion of the right and left mandibular processes.

Lateral fusion of maxillary and mandibular processes

At 11.5 days p.c. the fusion on the lateral aspect of the maxillary and mandibular processes has been initiated. In the area before the epithelia have come into contact syndecan-1 is expressed in the epithelium and in the mesenchyme adjacent to the future fusion area. Where the epithelia have come into contact a distinct line of syndecan-1 expression is present on the apical surface of the epithelia. This line is continuous with the dermal epithelial syndecan-1 expression, but does not extend to the oral epithelium. Syndecan-4 also shows a line of apical epithelial expression, which is absent once the epithelia have come in contact. Mesenchymal syndecan-1 expression is limited to the dermal side of the fusion area, while mesenchymal syndecan-4 expression is noted on the dermal side and on the fusion side. Mesenchymal fibronectin expression is strongest on the dermal side. Once the mesenchyme has become continuous syndecan-1 , syndecan-4 and fibronectin expression are noted on the dermal side of the mesenchyme and in the case of syndecan-1 and syndecan-4 in the dermal epithelium. No persistent band of syndecan-4 or fibronectin expression across the line of fusion, such as was seen after midline fusion, is noted here.

There is a differential pattern of expression of syndecan-1 and syndecan-4 in association with this fusion event, suggestive of functional significance. Before fusion takes place both syndecan-1 and syndecan-4 are expressed on the apical surface of the future fusion

epithelium and in the underlying mesenchyme. During the actual fusion event syndecan-1 and syndecan-4 show an opposite expression pattern, with syndecan-1 present and syndecan-4 absent in the epithelium at the line of fusion and syndecan-1 absent and syndecan-4 present in the underlying mesenchyme. During later stages of development (12.5 &13.5 days p.c.) stronger mesenchymal expression of syndecan-1 is seen in the mesenchyme adjacent to the future fusion area. Otherwise the progression of events is similar to that seen at 11.5 days p.c.

Some similarity is noted with the syndecan-1 expression during secondary palate formation, with loss of epithelial syndecan-1 expression at the line of fusion (Brinkley et al., 1992). Differences are noted between the fusion of the lateral aspect of the maxillary and mandibular processes (lateral fusion), the midline fusion of the right and left mandibular processes (mandibular midline fusion) and the fusion of the palatal shelves in the midline (palatal fusion). The persistent expression of syndecan-4 and fibronectin across the line of fusion seen in mandibular fusion is not noted in the lateral fusion event. The final stage of mandibular midline fusion and palatal fusion do not feature the strong mesenchymal syndecan-1 expression seen in lateral fusion. Further study is needed on the expression of syndecan-4 during palatal fusion. Based on the findings in this study and on the literature, mandibular midline fusion, lateral fusion and palatal fusion appear to be distinct processes with different distributions of molecular markers.

Palatal shelf formation and elevation

At 11.5 days p.c. increased expression of syndecan-1 and syndecan-4 is noted in the epithelium and mesenchyme of the developing lateral palatal shelves. At 12.5 days p.c. the syndecan-1 staining in this area remains similar. A thickening of the epithelium has formed at the lateral side of the developing lateral palatal shelves, which shows a distinct apical line

of syndecan-4 expression with minimal expression in the underlying mesenchyme. At this location the epithelium has changed from simple cuboidal to stratified columnar. At 13.5 days p.c. strong epithelial expression of syndecan-1 is noted in this area, with minimal expression in the underlying mesenchyme. The syndecan-4 staining is very strong on the apical surface of the epithelium at the lateral edge of the developing palatal shelves, with minimal expression in the underlying mesenchyme. The medial mesenchyme of the developing lateral palatal shelves shows a mild band of increased syndecan-4 expression. By 14.5 days p.c. the palatal shelves have elevated above the tongue. *In vivo* they will immediately be in contact with each other in the midline (Diewert and Tait, 1979). Moderate syndecan-1 and syndecan-4 expression is present on the epithelium of the elevated palatal shelves. The expression pattern of syndecan-1 observed here is similar to what has been found previously (Brinkley et al., 1992).

The process of the formation and elevation of the palatal shelves displays an intricate pattern of epithelial syndecan-1 and syndecan-4 expression. Epithelial syndecan-1 expression and epithelial syndecan-4 expression are noted early on and throughout the growth and elevation of the palatal shelves. Both syndecan-1 and syndecan-4 could function in the initiation, growth and elevation of the lateral palatal shelves. The localization of syndecan-4 in the lateral aspect of the shelf epithelium is suggestive of a strut function during the actual elevation process. This is compatible with the ability of the syndecans to associate (presumably indirectly, Miettinen and Jalkanen, 1994) with the actin cytoskeleton. The curvature that occurs as a result of epithelial cell shape and packing in this area has been suggested to serve to direct palatal expansion, resulting in palatal shelf elevation (Brinkley et al., 1992). The distinctive expression patterns of syndecan-1 and especially syndecan-4 in this curvature reemphasize the potential significance of this curvature in the lateral palatal shelf elevation process.

Formation of Meckel's cartilage

The earliest indication of the development of Meckel's cartilage are two elliptical areas of slightly increased syndecan-1 and moderately increased syndecan-4 expression and decreased fibronectin expression adjacent to the midline at 11.5 days p.c. At 12.5 days p.c. syndecan-1 expression has become stronger, while syndecan-4 expression has become weaker. In the midline in the anterior part of Meckel's cartilage primordium and in the posterior part slightly increased fibronectin expression is noted, with fibronectin expression still reduced in the intermediate parts of Meckel's cartilage. At 13.5 and 14.5 days p.c. Meckel's cartilage shows syndecan-1 and syndecan-4 expression only in a narrow band in the adjacent mesenchyme. Fibronectin expression is becoming strong in the outer layer of Meckel's cartilage and in the surrounding mesenchyme.

First syndecan-4 and then syndecan-1 are present in the pre-chondrogenic mesenchymal condensations of Meckel's cartilage. Fibronectin shows reduced expression in these areas as compared to the surrounding mesenchyme. As actual cartilage formation is being initiated first syndecan-4 and then syndecan-1 expression disappear from the center of Meckel's cartilage and become limited to the surrounding mesenchyme. At this point fibronectin expression increases in the developing Meckel's cartilage, especially in the outer ring. Studies on limb development have shown that syndecan-1 expression becomes reduced in chondrogenic foci. This is similar to what is seen in the formation of Meckel's cartilage.

Further experiments are needed to test if the initial upregulation of syndecan-4 and syndecan-1 followed by reduced expression are required for chondrogenesis to be initiated. *In vitro* culture of the mouse first branchial arch explant in combination with experimental

perturbation of syndecan-1 and/or syndecan-4 expression could be used (Slavkin et al., 1989; Slavkin et al., 1990a). Transfection with sense or anti-sense syndecan-1 and syndecan-4 constructs could be performed to increase or decrease expression. The antisense approach to reduce syndecan expression has been used successfully on cultured epithelial cells by stable transfection of a syndecan-1 antisense construct under a beta-actin promoter (Saunders et al., 1989b). Alternatively, the cultures could be treated with purified truncated forms of syndecan-1 and syndecan-4 core proteins, or with specifically constructed heparinsulfate fragments to dissect out which part of the GAG chains play a role during cartilage formation.

Another targeted means of affecting transcription of individual syndecan family members is the addition of anti-sense oligodeoxynucleotides (Slavkin, 1995). Anti-sense oligodeoxynucleotides have been very effective in the *in vitro* mandibular explant system in abrogating the transcription of EGF (Hu et al., 1992; Shum et al., 1993). The application of antisense oligodeoxynucleotides provides a relatively simple means of manipulating gene expression without interfering with previous stages of embryonic development. It has been shown that e.g. growth factors such as TGF β act in early stages of development during gross patterning (axis formation) and later on also function in the local development of anatomic structures (Mahmood et al., 1992).

Formation of mandibular bone

As early as 12.5 days p.c. an area of increased fibronectin expression is noted at a location where mandibular bone will develop later on, directly below and lateral to the developing Meckel's cartilage (Plate 3e). At 13.5 and 14.5 days p.c. fibronectin expression steadily increases in bone forming areas, especially in newly formed bone spicules (Plates 5c, 6c). Syndecan-1 expression remains low and syndecan-4 expression remains mild in areas of

bone formation. Fibronectin expression is low around the inferior alveolar neurovascular bundle, with strong expression in the actual vessel walls.

During the initiation and progression of bone formation syndecan-1 and syndecan-4 do not show a particularly distinctive pattern. The expression pattern of fibronectin however, is suggestive of a functional role in this process. There is strong experimental evidence that this is indeed the case for fibronectin, based on *In vitro* culture of primary rat calvarial osteoblasts in the presence and absence of anti-fibronectin antibodies (Moursi et al., 1996).

Tooth formation

The earliest indication of the initiation of molar formation in this investigation is the epithelial thickening seen at 10.5 days p.c. in the maxillary process. This thickening displays reduced syndecan-1 expression and increased syndecan-4 expression as compared to the other oral epithelium, with moderate syndecan-1 expression and mild syndecan-4 expression in the underlying mesenchyme. At 11.5 days p.c. the syndecan-1 expression remains similar, while the epithelial syndecan-4 expression decreases to the degree of staining of the underlying mesenchyme. At 12.5 days p.c. syndecan-1 and syndecan-4 show mild expression in the invaginating epithelial molar bud. Syndecan-1 shows increased and syndecan-4 and fibronectin show decreased expression in the surrounding mesenchyme. At 13.5 days p.c. syndecan-1 shows minimal expression in epithelium of developing molar cap, with increased expression in surrounding mesenchyme. Syndecan-4 shows a distinct line of expression in the dental lamina, with minimal expression in epithelium of developing molar cap, or in surrounding mesenchyme. Fibronectin shows slightly increased expression in surrounding mesenchyme. At 14.5 days p.c. mild syndecan-1 and syndecan-4 expression is seen in the core of the molar cap epithelium. The

surrounding mesenchyme shows mild syndecan-1 and minimal syndecan-4 and fibronectin expression.

The earliest indication of the initiation of incisor formation in this investigation are the two circular areas of syndecan-4 expression directly adjacent to the midline in the anterior mesenchyme of the mandibular process at 11.5 days p.c. More anterior sections would have shown the developing epithelial incisor cap. At 12.5 days p.c. strong syndecan-1 expression is noted in the incisor cap and in the surrounding mesenchyme. Syndecan-4 expression is present in the incisor cap and syndecan-4 and fibronectin expression are reduced in the surrounding mesenchyme. At 13.5 days and 14.5 days p.c. syndecan-1 expression has become mild in the epithelial enamel organ with strong expression in the surrounding mesenchyme. Increased syndecan-4 expression is noted in the core of the epithelial enamel organ as well as in the surrounding mesenchyme. Fibronectin expression is weak in the surrounding mesenchyme.

The observed patterns of syndecan-1 expression are similar to what has been described previously (Thesleff et al., 1988; Vainio et al., 1989; Vainio et al., 1991; Vainio and Thesleff, 1992a and 1992b). There are instances where other investigators noted strong syndecan-1 expression, for example in the invaginating epithelial molar bud at 12 days p.c. (Thesleff et al., 1988), where only mild expression is noted here. Differences in embedding and staining technique, such as incubation and washing conditions, may account for this difference. Despite the frequent use of quantitative terms such as mild, moderate, strong, it is important to keep in mind that immunohistochemstry is primarily a qualitative means of investigation. The distribution of expression and the relative changes in expression are similar to those found in previous investigations.

Though the timing of the events during incisor and molar formation differ somewhat, the observed expression patterns of syndecan-1 , syndecan-4 and fibronectin are very similar. By 11 days p.c. incisor tooth organs are at late cap stages, whereas molars are at their initial stage of tooth development. Therefore the initial stages of incisor formation are not included in these studies. The described pattern is highly suggestive of functional significance of syndecan-1 and syndecan-4 expression during incisor and molar formation. The absence of syndecan-1 , and the presence of syndecan-4 in the early epithelial thickening could be significant for the initiation of molar bud development. The presence of both syndecan-1 and syndecan-4 in the molar bud epithelium during later stages could be significant for further morphogenesis and differentiation. During the bell stage of incisor development syndecan-1 decreases and syndecan-4 expression increases in the enamel organ. The presence of syndecan-1 in the mesenchyme starting at the bud stage of development could be significant for the events leading to dentin formation, or could play a role in inhibiting bone formation (see section on mandibular bone formation).

Further study is needed to determine the syndecan-4 expression in relation to syndecan-1 expression during the earlier stages of incisor formation. The functional significance of the observed expression patterns could be tested using *in vitro* culture of developing tooth buds (Vainio et al., 1989), combined with experimental perturbation as described in the section on cartilage formation.

Formation of oral and buccal vestibulum

At 12.5 days p.c. the formation of the buccal and oral vestibulum has been initiated. From then on and through 14.5 days p.c. increased syndecan-1 and decreased syndecan-4 expression is noted in the epithelial groove adjacent to the posterior part of the tongue, and in the epithelial fold on the lateral side of the oral cavity. The mesenchyme surrounding

these epithelia shows low syndecan-1 expression and increased syndecan-4 and fibronectin expression.

The specific distribution of syndecan-1 , syndecan-4 and fibronectin is highly suggestive of functional involvement with this morphological event. Syndecan-1 may be highly expressed in the epithelium, because of the invaginating nature of this epithelium, analogous to tooth bud development (Thesleff et al., 1988). A distinct difference with tooth development however is the absence of syndecan-1 expression in the surrounding mesenchyme. Another difference is the absence of syndecan-4 in the invaginating epithelium, with strong presence of syndecan-4 in the surrounding mesenchyme. *In vitro* culture of this section of epithelium and its surrounding mesenchyme, in combination with experimental perturbation as described in the section on cartilage development, could be attempted to assess the functional significance of these expression patterns.

Concluding remarks

This investigation has focused on the distribution patterns of the proteoglycans (PGs) syndecan-1 and syndecan-4, and on a putative ligand of syndecan-1, fibronectin. The results show that these two members of the syndecan family of cell surface proteoglycans display distinctive spatial and temporal patterning during the embryonic development of the mouse first branchial arch. Therefore the hypothesis (page 19) is hereby accepted. As described above, there are instances where syndecan-1 is expressed in similar tissues as fibronectin, as well as instances where their expression patterns are opposite. This is also the case for syndecan-4 and fibronectin. The functional significance of the observed expression patterns still needs to be elucidated. An important point is that the

glycosaminoglycan (GAG) side chains, which are thought to represent the functional component of the PGs, display spatial and temporal differences in their composition. Therefore the same syndecan core protein may carry different side chains at different locations or at different times during development, with potentially significant effects on its function. Further investigations on the GAG side chains of the syndecans and the interactions with their ligands are needed to clarify the significance of the observed expression patterns presented here.

References

Abbott BD and Pratt RM (1991). Retinoic acid alters epithelial differentiation during palatogenesis. J. Craniofac. Genet. Dev. Biol. 11, 315-25.

Adams JC and Watt FM (1993). Regulation of development and differentiation by the extracellular matrix. Development 117, 1183-98.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994). Molecular biology of the cell (3rd ed.).Chapter 21: Cellular mechanisms of development. Garland Publishing, Inc., New York, NY.

Amaya E, Stein PA, Musci TJ, Kirschner MW (1993). FGF signaling in the early specification of mesoderm in Xenopus. Development 118, 477-87.

Baciu P, Acaster C, Goetinck PF (1993). Temporal and spatial expression of syndecan-4 during avian embryonic development. Supplement to Mol. Biol. Cell 4, 413a.

Beddington RSP (1982). An autoradiographic analysis of tissue potency in different regions of the embryonic ectoderm during gastrulation in the mouse. J. Embryol. Exp. Morphol. 69, 265-85.

Bernfield M and Hooper KC (1991). Possible regulation of FGF activity by syndecan, an integral membrane heparan sulfate proteoglycan. Ann. NY Acad. Sci. 638, 182-94.

Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ (1992). Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. Annu. Rev. Cell Biol. 8, 365-93.

Brinkley L, Morris-Wiman J, Bernfield M (1992). The distribution of syndecan during murine secondary palate morphogenesis. J. Craniofac. Genet. Dev. Biol. 12, 82-9.

Brown KS, Yamada Y, Abramczuk J, Kimata K (1991). New genetic approaches to craniofacial growth and malformation in the mouse. J. Craniofac. Genet. Dev. Biol. 11, 357-65.

Carey DJ, Evans DM, Stahl RC, Asundi VK, Conner KJ, Garbes P, Cizmeci-Smith G (1992). Molecular cloning and characterization of N-syndecan, a novel transmembrane heparan sulfate proteoglycan. J. Cell Biol. 117(1), 191-201.

Chernousov MA, Carey DJ (1993). N-syndecan (syndecan-3) from neonatal rat brain binds basic fibroblast growth factor. J. Biol. Chem. 268, 16810-4.

Chun JS and Bernfield M (1993). Syndecan-1 and integrin α5β1 appear to associate physically in the absence of their ligand fibronectin. Supplement to Mol. Biol. Cell 4, 283a.

Corless CL, Mendoza A, Collins T, Lawler J (1992). Co-localization of thrombospondin and syndecan during murine development. Developmental Dynamics 193, 346-58.

Damsky CH and Werb Z (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Current Opinion in Cell Biol. 4, 772-81.

Damsky C, Sutherland A, Fisher S (1993) Adhesive interactions in early mammalian embryogenesis, implantation and placentation. FASEB J. 7:1320-9.

David G, Van der Schueren B, Marynen P, Cassiman JJ, Van den Berghe H (1992). Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. J. Cell Biol. 118, 961-9.

David G, Bai XM, Van der Schueren B, Marynen P, Cassiman JJ, Van den Berghe H (1993). Spatial and temporal changes in the expression of fibroglycan (syndecan-2) during mouse embryonic development. Development 119, 841-54.

Diewert V and Tait B (1979). Palatal process movement in the rat as demonstrated in frozen sections. J. Anat. 128, 609-18.

Elenius K, Maatta A, Salmivirta M, Jalkanen M (1992). Growth factors induce 3T3 cells to express bFGF-binding syndecan. J. Biol. Chem. 267, 6435-41.

Elenius K and Jalkanen M (1994). Function of the syndecans - a family of cell surface proteoglycans. J. Cell Science 107, 2975-82.

Fitchett JE, McAlmon KR, Hay ED, Bernfield M (1990). Epithelial cells lose syndecan prior to epithelial-mesenchymal transformation in the developing rat palate. J. Cell Biol. 111, 145a.

Flaumenhaft R, Moscatelli D, Rifkin DB (1990). Heparin and heparan sulfate increase the radius of diffusion and action of basic fibroblast growth factor. J. Cell Biol. 111, 1651-9.

Gallo RL, Siebert E, Bernfield M (1993). Members of the syndecan family of heparan sulfate proteoglycans show distinct expression patterns during mouse development. Supplement to Mol. Biol. Cell 4, 413a.

Gould SE, Upholt WB, Kosher RA (1992). Syndecan-3: a member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chicken limb cartilage differentiation. Proc. Natl. Acad. Sci. 89, 3271-5.

Granstrom G, Kullaa-Mikkonen A, Zellin G (1990). Malformations of the maxillofacial region induced by retinoids in an experimental system. Int. J. Oral Maxillofac. Surg. 19, 167-71.

Gronowicz GA, DeRome ME, McCarthy MB (1991). Glucocorticoids inhibit fibronectin synthesis and messenger ribonucleic acid levels in cultured fetal rat parietal bones. Endocrinology 128, 1107-14.

Hall BK and Miayake T (1992). The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. Anat. Embryol. 186, 107-124.

Hayashi K, Hayashi M, Jalkanen M, Firestone M, Trelstad R, Bernfield M (1987). Immunocytochemistry of cell surface heparan sulfate proteoglycan in mouse tissues: a light and electron microscopic study. J. Histochem. Cytochem. 35, 1079-88.

Heino J (1993). Integrin-type extracellular matrix receptors in cancer and inflammation. Ann. Med. 25, 335-42.

Hinkes MT, Goldberger OA, Neumann PE, Kokenyesi R, Bernfield M (1993). Organization and promoter activity of the mouse syndecan-1 gene. J. Biol. Chem. 268, 11440-8.

Hirsch MR, Valarche I, Deagostini-Bazin H, Pernelle C, Joliot A, Goridis C (1991). An upstream regulatory element of the NCAM promoter contains a binding site for homeodomains. FEBS Letters 287(1,2), 197-202.

Hu CC, Sakakura Y, Sasano Y, Shum L, Bringas P, Jr., Werb Z, Slavkin HC (1992). Endogenous epidermal growth factor regulates the timing and pattern of embryonic mouse molar tooth morphogenesis. Int. J. Dev. Biol. 36, 505-16.

Huhtala P, Humphries MJ, McCarthy JB, Tremble PM, Werb Z, Damsky CH (1995). Cooperative signaling by alpha 5 beta 1 and alpha 4 beta 1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. Journal of Cell Biology, 129(3):867-79.

Hunt P and Krumlauf R (1992). Hox codes and positional specification in vertebrate embryonic axes. Annu. Rev. Cell Biol. 8, 227-56.

Jabs EW, Muller U, Li X, Ma L, Luo W, Haworth IS, Klisak I, Sparkes R, Warman ML, Mulliken JB, Snead ML, Maxson R (1993). A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. Cell 75, 443-50.

Jabs EW, Li X, Scott AF, Meyers G, Chen W, Eccles M, Mao J, Charnas LR, Jackson CE, Jaye M (1994). Jackson-Weiss and Crouzon syndromes are allelic with mutations in fibroblast growth factor receptor 2. Nature Genetics 8, 275-9.

Jalkanen M, Nguyen H, Rapraeger A, Kurn N, Bernfield M (1985). Heparan sulfate proteoglycans from mouse mammary epithelial cells: localization on the cell surface with a monoclonal antibody. J. Cell Biol. 101, 976-84.

Jalkanen M, Rapraeger A, Saunders S, Bernfield M (1987). Cell surface proteoglycan of mouse mammary epithelial cells is shed by cleavage of its matrix-binding ectodomain from its membrane-associated domain. J. Cell Biol. 105, 3087-96.

Jalkanen M, Mali M, Harkonen P, Leppa S (1990). Acquired epithelial phenotype for transformed mouse mammary tumor cells. J. Cell Biol. 111, 145a.

Jessel TM and Melton DA (1992). Diffusible factors in vertebrate embryonic induction. Cell 68, 257-70.

Johnston MC and Bronsky PT (1991a). Embryonic craniofacial development. In: The morphogenesis of Down Syndrome, 99-115. Wiley-Liss, Inc.

Johnston MC and Bronsky PT (1991b). Animal models for human craniofacial malformations. J. Craniofac. Genet. Dev. Biol. 11, 227-91.

Jones FS, Prediger EA, Bittner DA, De Robertis EM, Edelman GM (1992). Cell adhesion molecules as targets for Hox genes: neural cell adhesion molecule promoter activity is modulated by co-transfection with Hox-2.5 and -2.4. Proc. Natl. Acad. Sci. 89, 2086-90.

Jones MC (1990). The neurocristopathies: reinterpretation based upon the mechanism of abnormal morphogenesis. Cleft palate J. 27(2), 136-40.

Kato M and Bernfield M (1990). Epithelial cells made deficient in syndecan lose cell surface expression of E-cadherin and β1-integrins. J. Cell Biol. 111, 263a.

Kato M, Turnbull J, Hooper K, Gallagher J, Bernfield M (1991). Heparan sulfate chains on syndecan show cell type-specific fine structure. J. Cell Biol. 115, 125a.

Kaufman MH (1992). The atlas of mouse development. Academic Press, Inc., San Diego, CA.

Kessel M, Balling R, Gruss P (1990). Variations of cervical vertebrae after expression of a hox-1.1 transgene in mice.

Kim C, Goldberger OA, Gallo RL, Bernfield M (1994). Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue- and development-specific patterns. Mol. Biol. Cell 5, 797-805.

Kintner C (1992). Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. Cell 69, 225-36.

Kirby ML, Gale TF, Stewart DE (1983). Neural crest cells contribute to aorticopulmonary septation. Science 220, 1059-61.

Kojima T, Shworak NW, Rosenberg RD (1992). Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. J. Biol. Chem. 267, 4870-7.

Lammer EJ, Chen DT, Hoar RM, Agnish ND, Benke PJ, Braun JT, Curry CJ, Fernhoff PM, Grix AW, Lott IT, Richard JM, Sun SC (1985). Retinoic acid embryopathy. The New England J. of Med. 313(14), 837-41.

Liu YH, Kundu R, Wu L, Luo W, Ignelzi MA, Jr., Snead ML, Maxson RE, Jr. (1995). Premature suture closure and ectopic cranial bone in mice expressing Msx 2 transgenes in the developing skull. Proc. Natl. Acad. Sci. 92, 6137-41.

Mahmood R, Flanders K, Morris-Kay GM (1992). Interactions between retinoids and TGF beta's in mouse morphogenesis. Development 115, 67-74.

Marynen P, Zhang J, Cassiman JJ, Van den Berghe H, David G (1989). Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan proteoglycans of lung fibroblasts. J. Biol. Chem. 264, 7017-24.

Miettinen HM and Jalkanen M (1994). The cytoplasmic domain of syndecan-1 is not required for association with the detergent-insoluble cytoskeleton. J. Cell Sci. 107, 1571-81.

Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, Derynck R (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature, 376(6538), 337-41.

Morris-Kay G and Tuckett F (1991). Early events in mammalian craniofacial morphogenesis. J. Craniofac. Genet. Dev. Biol. 11, 181-91.

Morris-Kay G (1993). Retinoic acid and craniofacial development: molecules and morphogenesis. BioEssays 15(1), 9-15.

Moursi AM, Damsky CH, Lull J, Zimmerman D, Doty SB, Aota S, Globus RK (1996). Fibronectin regulates calvarial osteoblast differentiation. J. Cell Science 109, 1369-80.

Muenke M, Schell U, Hehr A, Robin NH, Losken HW, Schinzel A, Pulleyn LJ, Rutland P, Reardon W, Malcolm S, Winter RM (1994). A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome. Nature Genetics 8, 269-74.

Noden DM (1991a). Vertebrate craniofacial development: the relation between ontogenic process and morphological outcome. Brain Behav. Evol. 38, 190-225.

Noden DM (1991b). Cell movements and the control of patterned tissue assembly during craniofacial development. J. Craniofac. Genet. Dev. Biol. 11, 192-213.

Nurcombe V, Ford MD, Wildschut JA, Bartlett PF (1993). Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. Science 260, 103-6.

Prasthofer T, Ek B, Ekman P, Owens R, Hook M, Johansson S (1995). Protein kinase C phosphorylates two of the four known syndecan cytoplasmic domains *in vitro*. Biochem. Mol. Biol. Int. 36(4), 793-802.

Rapraeger A and Bernfield M (1982). An integral membrane proteoglycan is capable of binding components of the cytoskeleton and the extracellular matrix. In: Extracellular matrix 265-69. Hawkes S, Wang J (eds.), New York, Academic.

Rapraeger A, Jalkanen M, Bernfield M (1986). Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. J. Cell Biol. 103, 2683-96.

Rapraeger A, Jalkanen M, Bernfield M (1987). Integral membrane proteoglycans as matrix receptors: role in cytoskeleton and matrix assembly at the epithelial cell surface. In: Biology of extracellular matrix: a series. Biology of proteoglycans 2, 129-54. Wight TN, Mecham RP (eds.), Orlando, Academic.

Rapraeger A (1989). Transforming growth factor (type beta) promotes the addition of chondroitin sulfate to the cell surface proteoglycan (syndecan) of mouse mammary epithelia. J. Cell Biol. 109, 2509-18.

Reardon W, Winter RM, Rutland P, Pulleyn LJ, Jones BM, Malcolm S (1994). Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. Nature Genetics 8, 98-103.

Salmivirta M, Elenius K, Vainio S, Hofer U, Chiquet-Ehrismann R, Thesleff I, Jalkanen M (1991). Syndecan from embryonic tooth mesenchyme binds tenascin. J. Biol. Chem. 266(12), 7733-9.

Salmivirta M and Jalkanen M (1995). Syndecan family of cell surface proteoglycans: developmentally regulated receptors for extracellular effector molecules. Experientia 51, 863-72.

Sanderson R, Lalor P, Bernfield M (1989). B lymphocytes express and lose syndecan at specific stages of differentiation. Cell Regul. 1, 27-35.

Sanderson R, Sneed T, Young L, Sullivan G, Lander A (1992). Adhesion of B lymphoid (MPC-11) cells to type I collagen is mediated by the integral membrane proteoglycan, syndecan. J. Immun. 148, 3902-11.

Sanderson RD, Turnbull JE, Gallagher JT, Lander AD (1994). Fine structure of heparan sulfate regulates syndecan-1 function and cell behavior. J. Biol. Chem. 269, 13100-6.

Satokata I and Maas R (1994). Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nature Genetics 6, 348-55.

Saunders S, Jalkanen M, O'Farrel S, Bernfield M (1989a). Molecular cloning of syndecan, an integral membrane proteoglycan. J. Cell Biol. 108, 1547-65.

Saunders S, Nguyen H, Bernfield M (1989b). Mammary epithelial cells transfected with antisense cDNA reduce cell surface syndecan and become fibroblastic in morphology. J. Cell Biol. 109, 5a.

Serbedzija GN, Bronner-Fraser M, Fraser SE (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. Development 116, 297-307.

Shiang R, Thompson LM, Zhu YZ, Church DM, Fielder TJ, Bocian M, Winokur ST, Wasmuth JJ (1994). Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. Cell 78, 335-42.

Shum L, Sakakura Y, Bringas P, Jr., Luo W, Snead ML, Mayo M, Crohin C, Millar S, Werb Z, Buckley S, Hall FL, Warburton D, Slavkin HC (1993). EGF abrogation-induced fusilli-form dysmorphogenesis of Meckel's cartilage during embryonic mouse mandibular morphogenesis *in vitro*. Development 118, 903-17.

Slavkin HC, Bringas P, Jr, Sasano Y, Mayo M (1989). Early embryonic mouse mandibular morphogenesis and cytodifferentiation in serumless, chemically defined medium: a model for studies of autocrine and/or paracrine regulatory factors. J. Craniofac. Genet. Dev. Biol. 9, 185-205.

Slavkin HC, Sasano Y, Kikunaga S, Bessem C, Bringas P, Jr, Mayo M, Luo W, Mak G, Rall L, Snead ML (1990a). Cartilage, bone and tooth induction during early embryonic mouse mandible morphogenesis using serumless, chemically-defined medium. Connective Tissue research 24, 41-51.

Slavkin HC (1990b). Regulatory issues during early craniofacial development: a summary. Cleft Palate J.27(2), 101-9.

Slavkin HC(1991). Molecular determinants during dental morphogenesis and cytodifferentiation: a review. J. Craniofac. Genet. Dev. Biol. 11, 338-49.

Slavkin HC, Shum L, Bringas P, Jr., Werb Z (1992). Endogenous EGF regulates embryonic mouse mandibular morphogenesis *in vitro* using serumless, chemically-defined medium. In: The biological mechanisms of tooth movement and craniofacial adaptation, 37-45. Davidovitch Z (ed.), The Ohio State University College of Dentistry, Columbus, Ohio, U.S.A.

Slavkin HC (1995). Antisense oligonucleotides: an experimental strategy to advance a causal analysis of development. Int. J. Dev. Biol. 39, 123-6.

Slavkin HC (1996). Meeting the challenges of craniofacial-oral-dental birth defects. JADA 127: 681-2.

Solursh M, Reiter RS, Jensen KL, Kato M, Bernfield M (1990). Transient expression of a cell surface heparan sulfate proteoglycan (syndecan) during limb development. Developmental Biology 140, 83-92.

Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA, Damsky CH (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes and Development, 9(15):1883-95.

Sutherland AE, Sanderson RD, Mayes M, Seibert M, Calarco PG, Bernfield M, Damsky CH (1991). Expression of syndecan, a putative low affinity fibroblast growth factor receptor, in the early mouse embryo. Development 113, 339-51.

Sutherland AE, Calarco PG, Damsky CH (1993). Developmental regulation of integrin expression at the time of implantation in the mouse embryo. Development 119, 1175-86.

Thesleff I, Jalkanen M, Vainio S, Bernfield M (1988). Cell surface proteoglycan expression correlates with epithelial-mesenchymal interaction during tooth morphogenesis. Dev. Biol. 129, 565-72.

Thesleff I, Vaahtokari A, Vainio S (1991). Advances in histological methods open up new perspectives for craniofacial growth research. Proc. Finn. Dent. Soc. 87(1), 23-32.

Thompson LD, Pantoliano MW, Springer BA (1994). Energetic characterization of the basic fibroblast growth factor-heparin interaction: Identification of the heparin binding domain. Biochemistry 33, 3831-40.

Thompson SA, Higashiyama S, Wood K, Pollitt NS, Damm D, McEnroe G, Garrick B, Ashton N, Lau K, Hancock N, Klagsbrun M, Abraham JA (1994). Characterization of sequences within heparin-binding EGF-like growth factor that mediate interaction with heparin. J. Biol. Chem. 269, 2541-9.

Trautman MS, Kimelman J, Bernfield M (1991). Developmental expression of syndecan, an integral membrane proteoglycan, correlates with cell differentiation. Development 111, 213-20.

Vaahtokari A, Vainio S, Thesleff I (1991). Associations between transforming growth factor beta-1 mRNA expression and epithelial-mesenchymal interactions during tooth development. Development 113, 985-94.

Vainio S, Jalkanen M, Thesleff I (1989). Syndecan and tenascin expression is induced by epithelial-mesenchymal interactions in embryonic tooth mesenchyme. J. Cell Biol. 108, 1945-54.

Vainio S, Jalkanen M, Vaahtokari A, Sahlberg C, Mali M, Bernfield M, Thesleff I (1991). Expression of syndecan is induced early, is transient and correlates with changes in mesenchymal cell proliferation during tooth organogenesis. Developmental Biology 147, 322-33.

Vainio S and Thesleff I (1992a). Coordinated induction of cell proliferation and syndecan expression in dental mesenchyme by epithelium: Evidence for diffusible signals. Dev. Dyn. 194, 105-17.

Vainio S and Thesleff I (1992b). Sequential induction of syndecan, tenascin and cell proliferation associated with mesenchymal cell condensation during early tooth development. Differentiation 50, 97-105.

Vainio S, Karavanova I, Jowett A, Thesleff I (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during tooth development. Cell 75, 45-58.

Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109, 877-89.

Wilkie AO, Slaney SF, Oldridge M, Poole MD, Ashworth GJ, Hockley AD, Hayward RD, David DJ, Pulleyn LJ, Rutland P, et al. (1995). Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. Nature Genetics 9, 165-72.

Wilkinson DG (1993). Molecular mechanisms of segmental patterning in the vertebrate hindbrain and neural crest. BioEssays 15(8), 499-505.

Wood A, Ashhurst DE, Corbett A, Thorogood P (1991). The transient expression of type II collagen at tissue interfaces during mammalian craniofacial development. Development 111, 955-68.

Woods A, Couchman JR (1992). Protein kinase C involvement in focal adhesion formation. J. Cell Sci. 101, 277-90.

Woods A and Couchman JR (1994). Syndecan-4 Heparan sulfate proteoglycan is a selectively enriched and widespread focal adhesion component. Mol. Biol. Cell 5, 183-92.

Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64, 841-8.