Chapter 1

Introduction
Introduction

The skull is one of the latest inventions of vertebrate evolution. It serves many very vital functions, like eating and defence. Moreover, it harbours the brain and many sense organs that are required for perception of the environment, e.g. for sight, smell, hearing and taste. In primates it has equipped the animal with a unique tool to express emotions. Unfortunately, the skull appears to be very susceptible to malformations. Many babies are born each year with craniofacial birth defects due to e.g. inappropriate development of the craniofacial primordia or due to abnormalities in neural tube development. Recent studies show that mutations in developmental genes appear to be the underlying cause of many of these birth defects.

In this introduction I will discuss the embryonic origin of most of the skull bones and give an overview of craniofacial morphogenesis. Moreover, I will discuss the roles of a number of genes important in craniofacial development. The chapter will conclude with the discussion of a number of well-known craniofacial defects.

The skull and its embryonic origins

The skull is one of the most complicated parts of the vertebrate body. It has long stimulated questions as to how it is constructed and how it develops during ontogeny. Anatomically the skull can be divided into brain case and the facial skeleton. The braincase consists of the frontal, parietal and supraoccipital bones, which overlie and protect the brain. The skull base supports the brain and consists of exo- and basioccipital and sphenoid bones and the nasal capsule (=ethmoid bone) (see Fig. 1.1). The facial skeleton is constituted by the nasal bones, the premaxillary, maxillary, zygomatic and squamosal bones and the mandible. The nasal and otic capsules are sensory organs that develop from the otic and nasal placodes, respectively (see Fig. 1.1).

A subdivision of the skull bones can also be made based upon their embryonic origin (Couly et al., 1993). The chordal skeleton consists of bones that are derived from cephalic and somitic mesoderm. The cephalic mesoderm surrounds the primitive brain vesicles and it condensates to form the supraoccipital bone, part of the otic capsule and the basisphenoid bones. On the other hand, mesoderm derived from the first five occipital somites forms the basi- and exooccipital bones and also some structures of the otic capsule (Couly et al., 1993).
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The prechordal (or achordal) skeleton consists of bones that are derived from the cranial neural crest. The neural crest is a population of cells that detaches from the neural folds during neural tube closure and undergoes an epithelial to mesenchymal transition. The cells migrate away from the neural tube and populate many regions in the embryo to give rise to various tissues. The cranial neural crest is derived from fore- mid- and hindbrain regions. It contributes to formation of many structures like connective tissue, odontoblasts and neurons and glia of cranial ganglia. Moreover, it forms a significant part of the skull. The basipresphenoid and more anterior bones comprising the entire facial skeleton are entirely neural crest derived, but also a part of the otic capsule (Couly et al., 1993).

The embryonic origin of the calvaria remains uncertain. Studies carried out by Couly et al. (1993) show that the frontal and parietal bones are neural crest derived, whereas other studies conclude that they are derived from cephalic mesoderm (Goodrich, 1930; LeLievre, 1978; Jarvik, 1980; de Beer, 1985; Noden, 1986). It should be kept in mind that all these studies were carried out using chick embryos, which are well accessible to tissue transplantation studies. However, the avian skull is considerably different from the mammalian skull and many structures along the neural crest:mesoderm interface are anatomically very different (e.g. vaults, palatine, sphenoid regions). Therefore, it is not clear if all avian data on the embryonic origins of certain skull bones can easily be extrapolated to the mammalian situation. A recently generated two component transgenic mouse line system, in which LacZ is expressed in the Wnt1 domain may shed more light on this issue (Chai et al., 2000).
**Craniofacial morphogenesis**

The face develops on the rostral end of the embryonic axis from the facial primordia that are arranged around the primitive mouth, the stomodeum. The primordia include the frontonasal process and the first branchial arch derived paired mandibular and maxillary processes (see Fig. 1.4). The second, third, fourth and sixth arch will contribute to more posterior structures of the larynx and some thoracic blood vessels. Early during craniofacial development, the frontonasal process and the branchial arches are populated with paraxial mesoderm and cranial neural crest cells (reviewed by Noden, 1988; Köntges and Lumsden, 1996; Couly et al., 1996).

**The branchial arches**

The branchial arches develop as bulges surrounding the pharynx (see Fig. 1.2). Within the pharynx, they are lined with endoderm and their outer surface is covered with ectoderm. Branchial pouches and branchial grooves demarcate their borders on the inner and outer surface of the pharynx, respectively. In between the arches the branchial membranes are formed at the boundary between endo- and ectoderm. The branchial arches are populated by paraxial mesoderm and neural crest cells (Couly et al., 1993). The paraxial mesoderm forms the craniofacial muscles, some skeletal elements and vascular tissues, while cranial neural crest cells contributes to the peripheral nervous system, connective tissues and cartilage (Le Douarin, 1982; Noden, 1988; Kimmel et al., 1991; see Fig. 1.2).

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**Fig. 1.2**: Schematic view of pharynx and branchial arches. Every branchial gives rise to a basic set of structures: a cartilage component, a nervous component, a vascular component and a muscular component.
The first branchial (or mandibular) arch is populated by neural crest cells derived from the midbrain and the three most anterior rhombomeres (see Fig. 1.3). It develops into a mandibular and a maxillary portion. Within the mandibular portion Meckel's cartilage is formed, which provides a template for subsequent development of the mandible. A substantial part of Meckel's cartilage will be covered by bone, but more proximal elements persist as two ear ossicles, the malleus and the incus, while its anterior extremity persists as a cartilage element connecting both dentaries. The musculature associated with the first branchial arch includes the muscles of mastication, which are innervated by the trigeminal nerve. The maxillary process of the mandibular arch will give rise to the upper jaw and cheek region (see Table 1).

The second branchial (or hyoid) arch is populated by rhombomere 3 and 4 derived neural crest cells (see Fig. 1.3). They condensate to form Reichert’s cartilage, most of which develops into the hyoid bone and stapes, the styloid process of the temporal bone and the stylohyoid ligament. The muscles formed by the second arch are the facial muscles, which are innervated by the facial nerve (see Table 1).

The third branchial arch is populated by crest derived from rhombomere 5 and 6 and forms the greater cornu and the lower part of the hyoid bone. The glossopharyngeal nerve innervates the third arch (see Fig. 1.3 and Table 1). The laryngeal cartilages seem to be formed independently of the neural crest, as they most likely derive from lateral plate mesoderm residing in the fourth and sixth branchial arches (Noden, 1986 and references therein). Innervation is brought about by the vagus nerve (see Table 1).

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**Fig 1.3:** Cranial neural crest migration into the frонтonasal process and the branchial arches. The origin of the neural crest in the frонтonasal process and branchial arches is color-coded. Abbreviations: BA, branchial arch; FNP, frонтonasal process; R: rhombomere, NC: neural crest (Figure from Le Douarin and Kalcheim, 1999).
The frontonasal process

The frontonasal process is populated by neural crest cells derived from fore- and midbrain regions (Osumi-Yamashita et al., 1994; see Fig. 1.3). They give rise to the trabecular cartilage, which originally arises as a pair of bar-like cartilages. Later it develops into the premaxillary bones, the ethmoid (comprising the nasal capsule and the nasal septum) and the presphenoid bones. Also the frontal and nasal bones develop from the frontonasal process (Rathke, 1839; reviewed by de Beer, 1931, 1937; see Table 1). There are a few reasons that suggest that the trabecula may represent a "premandibular" component of the facial skeleton and belong to the same segment as the ophtalmic nerve. These are its neural crest origin, its topographical location, its morphology and the metamerical organization of cranial nerves (Huxley, 1874; reviewed by Goodrich, 1930; de Beer, 1931, 1937; Stadmüller, 1936; Kuratani, 1997).
### Table 1: Structures developing in the branchial arches

<table>
<thead>
<tr>
<th>Arch</th>
<th>Skeletal elements</th>
<th>Nerve</th>
<th>Vasculature</th>
<th>Musculature</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0)</td>
<td>Trabecular cartilages (transient)</td>
<td>Ophthalmic branch of trigeminal nerve (V)</td>
<td></td>
<td>Muscles for mastication</td>
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<tr>
<td>Premandibular arch</td>
<td>Ethmoid (=nasal capsule)</td>
<td></td>
<td></td>
<td>Mylohyoid</td>
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<tr>
<td></td>
<td>Nasal bone</td>
<td></td>
<td></td>
<td>Tensor tympani</td>
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<td></td>
<td>Frontal bone</td>
<td></td>
<td></td>
<td>Tensor palatini</td>
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<td></td>
<td>Lacrymal bone</td>
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<td></td>
<td>Basiphenoid bone</td>
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<td></td>
<td>Premaxilla</td>
<td></td>
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<tr>
<td>1</td>
<td>Meckel's cartilage (transient)</td>
<td>Trigeminal (V)</td>
<td>Maxillary arteries</td>
<td>Muscles for facial expression</td>
</tr>
<tr>
<td>(Mandibular arch)</td>
<td>Malleus</td>
<td></td>
<td></td>
<td>Posterior belly of digastic</td>
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<tr>
<td></td>
<td>Incus</td>
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<td></td>
<td>Styloidus</td>
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<td></td>
<td>Mandible</td>
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<td>Styloid process</td>
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<td></td>
<td>Maxilla</td>
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<td></td>
<td>Zygomatic arch</td>
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<td>Palatine</td>
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<td>Alisphenoid</td>
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<td>Squamosal</td>
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<tr>
<td>2</td>
<td>Reichert's cartilage (transient)</td>
<td>Facial (VII)</td>
<td>Stapedial arteries (in embryo only)</td>
<td>Muscles for facial expression</td>
</tr>
<tr>
<td>(Hyoid arch)</td>
<td>Stapes</td>
<td></td>
<td></td>
<td>Posterior belly of digastic</td>
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<td></td>
<td>Styloid process</td>
<td></td>
<td></td>
<td>Styloidus</td>
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<td></td>
<td>Lesser cornu of hyoid</td>
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<td>Upper part of body of hyoid bone</td>
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<td>3</td>
<td>Greater cornu of hyoid</td>
<td>Glosso-pharyngeal (IX)</td>
<td>Common and internal carotid arteries</td>
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<td></td>
<td>Lower part of body of hyoid bone</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4-6</td>
<td>Thyroid, Cricoid, Arytheneoid, Corniculate and Cuneiform cartilages</td>
<td>Vagus (X)</td>
<td>Part of adult arch of aorta</td>
<td>Intrinsic muscles of the larynx</td>
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<td></td>
<td></td>
<td></td>
<td>Part of subclavian artery</td>
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<td></td>
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<td></td>
<td>Part of left pulmonary artery</td>
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<td></td>
<td></td>
<td></td>
<td>Ductus arteriosus</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Part of right pulmonary artery</td>
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</table>
Development of the face

Facial development starts with the emergence of the facial primordia, the branchial arches and the frontonasal process. They grow out by controlled proliferation of neural crest derived mesenchymal cells, which are dependent on signals from the overlying ectoderm (Wedden, 1987; Richman and Tickle, 1992). The primordia undergo complex morphogenetic interactions and ultimately they a complete face.

Development of lower and upper jaw and nose region

The mandibular and maxillary processes of the first arch grow out and develop in a ventromedial direction (see Fig. 1.4A-D and Table 1). The mandibular processes fuse in the midline and eventually form the lower jaw. The maxillary processes of the first arch give rise to the upper jaw and cheek regions (see Fig. 1.4A-D and Table 1). Early during facial development the surface ectoderm on lateral regions of the frontonasal process thickens and forms the nasal placodes (see Fig 1.4A). Due to outgrowth of the frontonasal process the placodes deepen, resulting in formation of the lateral and medial nasal processes (see Fig. 1.4B, C). The nasal processes fuse inferiorly with each other and with the outgrowing maxillary processes to form the intermaxillary segment (see Fig. 1.4C). The intermaxillary segment will form the primary palate, premaxilla and philtrum (see Fig. 1.4C and D, Fig. 1.5A, B, C and Table 1). The nasolacrimal groove is the cleft, which separates the lateral nasal and maxillary processes (see Fig. 1.4C and D).

Fig. 1.4: Development of the lower and upper jaw and nose region. Frontal views on cranial regions of developing embryos. Abbreviations: 2nd: 2nd branchial arch, 3rd: 3rd branchial arch, FNP: frontonasal process, FP: frontal process, He: heart bulge, IMS: intermaxillary segment; LNP: lateral nasal process, Mn: Mandibular process, MNP: medial nasal process, Mx: maxillary process; NLG: nasolacrimal groove, Npi: nasal pit, NPl: nasal placode; Ph: philtrum, St: stomodeum.
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Development of secondary palate and internal nose structures

From the inner walls of the maxillary arches palatal shelves grow out (see Fig. 1.5A). First they project vertically (Fig. 1.5D), but later they extend upward and fuse in the midline with each other. Anteriorly they fuse with the primary palate to form the secondary or definitive palate (see Fig. 1.5C, F). The nasal septum grows downwards from the frontal process and fuses with the palate (see Fig. 1.5D, E, F). The definitive palate physically separates nasal and oral cavities and the nasal septum separates both nasal cavities. The nasopalatine canal (incisive foramen) persists in the palatine midline between the premaxillary portion of the maxilla and the palatine processes of the maxilla (see Fig. 1.5C). Within the lateral walls of the nasal cavity the nasal conchae are formed and develop into the nasal labyrinth.


Genes involved in craniofacial development

Whilst a lot is known about origins of skull bones and craniofacial morphogenesis, relatively little is known about molecular mechanisms regulating craniofacial development. Craniofacial development is a multi-stage process. It involves the formation and migration of cranial neural crest cells, followed by the correct outgrowth of the facial primordia and the morphogenesis
and differentiation of the skeletal elements. During these stages, members of many gene families are expressed and have functions in the (presumptive) neural crest cells and in the development of the craniofacial primordia (review by Francis-West, 1998).

**Genes involved in prepatternning of the cranial neural crest**

Before craniofacial development starts, members of different gene families regionalize the neural tube. They are responsible for the identity of neural crest cells that originate from these regions. However, pre patterning of the neural tube alone does not bring about specification of neural crest identity. Environmental signals, such as signals emitted from the branchial arches or the somites, are also important (Grapin-Botton et al., 1995; Itasaki et al., 1996; Grapin-Botton et al., 1997; Hunt et al., 1998, Trainor and Krumlauf, 2000).

**Hox genes**

*Hox* genes are homeobox genes expressed from caudal neural tube regions up to a specific anterior border. The 'Hox-code', which is the combination of *Hox* genes expressed within a certain body segment, determines its AP identity. The most anterior *Hox* genes are expressed up to hindbrain regions. Their overexpression or loss-of-function mutations cause craniofacial abnormalities (see Fig. 1.6). *Hoxa1* and *Hoxb1* are transiently expressed in rhombomeres 4 to 6. *Hoxa1* and *Hoxb1* mutant mice display abnormalities in second branchial arch derivatives and the VIIth to XIth cranial nerves (Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Dollé et al., 1993; Mark et al., 1993; Goddard et al., 1996; Studer et al., 1996, 1998; Gavalas et al., 1998). *Hoxa2* is expressed in the developing hindbrain. In *Hoxa2* mutant mice, the identity of cranial neural crest cells in the second branchial arch seems to have changed into a first branchial arch identity (Gendron-Maguire et al., 1993; Rijli et al., 1993). *Hoxa3* is expressed up to the 5th rhombomere and mouse mutants display abnormalities in cartilages and bones of the jaw (Lufkin et al., 1991; Chisaka and Capecchi, 1991).

**Otx1, Otx2, Emx1, Emx2 and Gbx2**

The homeobox genes *Otx1* and -2 and *Emx1* and -2 are homologs of the *Drosophila* genes *orthodenticle* and *empty spiracles*, respectively. They are expressed in nested patterns in the fore- and midbrain territories indicative of the existence of a genetic code responsible for AP
patterning of these brain regions (see Fig. 1.6). Indeed, genetic analysis reveals a role for these genes in patterning these regions and craniofacial primordia.

*Otx2* is expressed in fore- and midbrain (see Fig. 1.6, Simeone et al., 1993; Ang et al., 1994). Inactivation or overexpression of *Otx2* causes abnormalities of brain development (Matsuo et al., 1995; Acampora et al., 1995; Pannese et al., 1995; Ang et al., 1996). *Otx2* heterozygous mutant mice display multiple skeletal defects in the prechordal skull. Homozygous *Otx2* mutant embryos die before E10.0 and lack structures corresponding to the rostral head (Matsuo et al., 1995).

*Otx1* is expressed in the more posterior forebrain regions and in the midbrain. *Otx1* mutants display morphological transformation of fore- and midbrain regions into hindbrain (see Fig. 1.6, Acampora et al., 1996). Moreover, *Otx1* is capable to take over functions of *Otx2* in patterning of the neural crest (Suda et al., 1999). *Emx1* and *Emx2* are expressed in overlapping patterns in the forebrain. *Emx1* and *Emx2* mutants display abnormalities in the telencephalic cortex (see Fig. 1.6, Qiu et al., 1996; Pellegrini et al., 1996; Yoshida et al., 1997).

*Gbx2* is expressed in regions of the anterior hindbrain with a sharp boundary at the mid-/hindbrain border, marking the isthmic organizer (see Fig. 1.6). *Gbx2* mutant mice display abnormal development of the anterior hindbrain and genetic markers of the isthmic organiser.
region have shifted. These genetic studies suggest a role for *Gb*2 in positioning the mid-
hindbrain boundary (Wassarman et al., 1997; Millet et al., 1999; Simeone, 2000).

Genes involved in neural crest migration
Neural crest cells migrate away in streams from the neural tube to reach their destination in the
facial primordia and branchial arches (see Fig. 1.2). The process of migration is regulated by a
several genes, two of which will be discussed below.

**AP-2**
The transcription factor *AP-2* is a retinoic acid responsive gene that is first expressed during
neural crest determination, migration and in the facial primordia (Mitchell et al., 1991; Shen et
al., 1997). In *AP-2* mutant mice, the cranial neural tube fails to close. Moreover, abnormalities
are found in the prechordal craniofacial skeleton (Schorle et al., 1996; Zhang et al., 1996).
TUNEL analysis revealed that neuroepithelial cells and late migrating cranial neural crest cells
are apoptotic (Schorle et al., 1996). Apparently *AP-2* is involved with survival of premigratory
and migratory cranial neural crest cells.

**PDGFαR**
Platelet derived growth factors (PDGFs) have been shown to regulate cell growth and survival,
but also cell morphology and movement (for review see Clesson-Welsh, 1994; Kazlauskas,
1994). Two PDGF receptors exist, PDGFαR and PDGFβR (Seifert et al., 1989). Mice with
mutations in *PDGFαR*, among which the Patch mouse (Ph), show a number of defects
comprising cleft faces and spina bifida (Grüneberg and Truslove, 1960; Soriano, 1997). The
cleft face phenotype has been associated with a defect in the migration and with apoptosis of
migrating neural crest cells (Morrison-Graham et al., 1992; Soriano, 1997).

Genes involved in patterning of the craniofacial primordia
Members of many gene families are expressed in the developing craniofacial primordia, both in
ectoderm and mesenchyme. Their outgrowth is dependent on proliferation of mesenchymal
cells, which is tightly controlled by signals from the overlying epithelium and branchial pouch
endoderm (Wedden, 1987; Richman and Tickle, 1992).
Fibroblast growth factor family (Fgf)

At least 15 members of the Fgf family and the Fgf receptors-1, -2 and -3 are expressed in the developing craniofacial primordia. The Fgf ligands are expressed in the epithelium, while their receptors are expressed by the underlying mesenchyme (Drucker and Goldfarb, 1993; Wall and Hogan, 1995; Hartung et al., 1997; Bachler and Neubüser, 2001). Fgf2 and Fgf4 coated beads were shown to support outgrowth of the frontonasal process and mandibular arch mesenchyme in chick embryos (Richman et al., 1997). Fgf8 is expressed by the epithelium of the nasal pits and the first branchial arches. Hypomorphic Fgf8 mutant and conditional Fgf8 mutant mouse embryos have craniofacial and branchial arch abnormalities (Meyers et al., 1998; Trumpp et al., 1999). In humans several craniofacial syndromes are known to be due to mutations in FGF receptors, such as many forms of craniosynostosis (reviewed by Muenke and Schell, 1995; Wilkie et al., 1995; Wilkie et al., 1997).

TGF-β superfamily

TGF-β molecules, bone morphogenetic proteins (Bmps) and activins belong to the superfamily of transforming growth factor β (TGF-β)-signalling proteins. Many members and their receptors are expressed by the craniofacial primordia (Pelton et al., 1989, Pelton et al., 1991; Millan et al., 1991). Mutations in some TGF-β isoforms result in craniofacial abnormalities in upper and lower jaws and palatal shelves (Sanford et al., 1997; Kaartinen et al., 1995; Proetzel et al., 1995). Bmp2 and -4 null mutants die before onset of craniofacial development. Haploinsufficient Bmp4 mutants are viable and have abnormalities in frontal and nasal bones (Winnier et al., 1995; Zhang and Bradley, 1996; Dunn et al., 1997). Bmp7 mutants have abnormalities in cranial bones and in the skull base (Dudley et al., 1995; Luo et al., 1995). The short ear mutant was shown to carry mutations in the Bmp5 gene (Kingsley et al., 1992).

Sonic hedgehog

Shh is a signaling protein that is related to the Drosophila gene hedgehog (hh). Mouse Shh is expressed in the craniofacial primordia. It is expressed by the ectoderm and endoderm of the first branchial arch and in the ectoderm of the nasal processes. Shh mutants have severe craniofacial defects. Most defects are secondary to the defective splitting of the eye field, but some are directly caused by mutations in Shh. In Shh mutants the first branchial arch degenerates after E9.5, resulting in malformation or absence of most first branchial arch
derivatives at birth (Chiang et al., 1996). In chick, overexpression and inhibition of Shh signalling also results in severe craniofacial abnormalities (Hu and Helms, 1999). Evidence suggests that Shh plays a role in regulation of cranial neural crest cell survival (Ahlgren et al., 1999). In humans, mutations in SHH are implicated in the etiology of holoprosencephaly (Belloni et al., 1996; Roessler et al., 1996).

**Retinoic acid receptor-α and -γ**

Several types of retinoic acid receptors (RARs) have been identified. RAR-α, -β and -γ and the retinoid X receptors, RXR-α, -β and γ. RAR-α and RAR-γ are strongly expressed by the frontonasal process and branchial arches during and following neural crest migration (Ruberte et al., 1990). In RAR-α/RAR-γ double mutant mouse skulls, all the structures derived from the frontonasal process were partially or completely absent (Lohnes et al., 1994; Mendelsohn et al., 1994). The frontal, nasal, premaxillary, ethmoid and presphenoid bones were largely missing. In contrast, the derivatives of the mandibular process of the first arch were present and only little affected. These abnormalities probably result from increased cell death in the frontonasal mesenchyme at E10.5. Therefore retinoic acid may be required for survival of post-migratory neural crest cells within the frontonasal process (Lohnes et al., 1994).

**Gli genes**

Members of the Gli family are zinc finger containing proteins that show significant sequence similarity to the product of the Drosophila segment polarity gene cubitus interuptus (ci) (Ruppert et al., 1988; Orenic et al., 1990; Hui et al., 1994). They are mediators of Shh signaling (Marigo et al., 1996; Lee et al., 1997). In both human and mouse, three closely related Gli genes exist, Gli1-3. During craniofacial development the Gli genes are expressed in the neural crest derived mesenchyme of the frontonasal process and branchial arches. Gli2 and Gli3 are also expressed in the migrating neural crest (Walterhouse et al., 1993; Hui et al., 1994). Mutation of Gli2 results in truncation of the distal part of the maxilla and mandible. Moreover, loss of the presphenoid, maxillary bone and palatine shelves causes clefting in the skull of these mice. In contrast, mutations in Gli3 result in an enlargement of the maxillary region. Furthermore, the nasal processes are smaller and some clefting occurs. In Gli2/Gli3 double mutants the abnormalities in the craniofacial region are enhanced (Mo et al., 1997). Mutation in Gli3 results in, amongst others, craniofacial abnormalities in the human syndrome
Greig's cephalopolysyndactyly (GCPS) and the extra toes (Xt) mouse (Johnson, 1967; Gollop and Fontes, 1985; Vortkamp et al., 1991, 1992; Schimmang et al., 1992; Hui and Joyner, 1993).

**Msx genes**

The homeobox containing Msx genes are expressed in migratory and cranial neural crest derived mesenchyme. They have important functions in epithelio-mesenchymal interactions (Hill et al., 1991; Robert et al., 1989; Mackenzie et al., 1991a,b; Suzuki et al., 1991; Mina et al., 1995). In Msx1 mutant mice all facial structures fail to develop normally and they lack teeth (Satokata and Maas, 1994). Human syndromes exist that are associated with mutations in Msx1 and Msx2. MSX1 haploinsufficiency results in loss of premolar and molar teeth, but the facial skeleton develops normally (Vastardis et al., 1996). A mutation in one copy of human MSX2, causes craniosynostosis (Liu et al., 1996; Ma et al., 1996).

**Dlx genes**

Dlx genes are homeobox genes that are related to the *Drosophila* gene Distalless (Dll). In mouse 6 Dll-related genes exist. They are expressed in migrating cranial neural crest and in the cranial neural crest derived mesenchyme or ectoderm overlying the nasal pits (Qiu et al, 1995; Qiu et al., 1997; Acampora et al., 1999; Depew et al, 1999; Thomas et al., 2000). Dlx1, -2 and -5 mutant mice have severe craniofacial abnormalities. Dlx2 mutants have abnormalities in proximal first and second arch derived structures, whereas Dlx1 mutants only have abnormalities in first arch derivatives (Qiu et al., 1995; Qiu et al., 1997). Dlx1/Dlx2 double mutants have similar abnormalities in both first and second arch derivatives as found in the single mutants. In addition, they lack the upper molars. Dlx5 mutants have abnormalities in ears, noses, lower jaw and calvaria (Depew et al., 1999; Acampora et al., 1999).

**aristaless-related genes**

*aristaless* (*al*) is a *Drosophila* gene that is expressed in central regions of the leg, eye and wing imaginal discs that grow out and become the most distal tips of the appendages (Schneitzi et al., 1993). It controls growth and differentiation of the tip of the leg (Campbell and Tomlinson, 1998). In vertebrates, a large group of genes related to *aristaless* exists. They can be categorized in three classes of which class I genes are predominantly expressed in
mesenchymal structures of limb and craniofacial regions as described in Chapter 2. Among them are *Alx4*, the gene that is defective in the Strong’s Luxoid mutant, *Prx1, Cart1, Prx2, Prx3* and *Alx3* (Meijlink et al, 1999).

*Alx3, Alx4* and *Cart1* are expressed by the frontonasal process and nasal processes, distally in the branchial arches and anteriorly in the limbs as described in Chapter 2. *Alx4* and *Cart1* single and double mutant mice were generated in other labs. In Chapter 3 the generation of the *Alx3* single and *Alx3/Alx4* double mutants is described. *Alx3* mutant mice do not have an obvious phenotype. In contrast, *Alx4* mutants have preaxial polydactyly and mild craniofacial abnormalities. The human syndrome symmetric parietal foramina (PFM) is also caused by mutations in the ALX4 gene (Mavrogiannis et al., 2001; Wuyts et al., 2000). *Cart1* mutant mice lack all skull vaults and have abnormalities in their skull base and facial skeleton (Zhao et al., 1995; Qu et al., 1997; Chapter 4 of this thesis). *Alx4/Cart1* and *Alx3/Alx4* double mutants have cleft nose regions and limb abnormalities (Qu et al., 1999; Beverdam et al., in press). These data, together with the results presented in Chapter 4 suggest that these three genes have overlapping functions in patterning the nasal processes and distal regions of the mandibular arch.

*Prx1* and *Prx2* are also expressed in overlapping patterns by the frontonasal process, nasal processes and in the branchial arches as described in Chapter 2. *Prx1* mutant mice were generated in E.N. Olson’s lab and *Prx2* single and *Prx1/Prx2* double mutant mice were generated both in our lab and by Lu and colleagues (ten Berge et al., 1998; Lu et al., 1999). *Prx2* mutant mice do not have abnormalities. In contrast, *Prx1* mutants display complex craniofacial phenotype including malformation of bones of the facial skeleton, the skull base, the otic capsule and second branchial arch derived structures (Martin et al., 1995). In *Prx1/Prx2* double mutants, the *Prx1* phenotype is enhanced suggesting overlapping roles for both genes in patterning of the mandibular and hyoid arch structures (ten Berge et al., 1998; Lu et al., 1999).

*Prx3* is expressed in foetal and adult brain, initially in broad areas that develop in the dorsal thalamus, pretectum and tectum. In the adult, the gene is most prominently expressed in nuclei that are part of the subcortical visual system (Van Schaick et al., 1997). In addition, it is
expressed by the craniofacial primordia and proximally in the limb buds (Chapter 2 of this thesis). Human Prx3 is a candidate gene for the Cornelia de Lange syndrome (De Lange, 1933; Blaschke et al., 1998; Semina et al., 1998). This syndrome is characterized by a combination of mental retardation, craniofacial features, eye defects and limb defects. SHOX is highly related to Prx3. The gene gives rise to at least two splice variants, SHOXA and SHOXB. Both are expressed in skeletal muscle and bone marrow fibroblasts. SHOXA is also expressed in placenta, heart and pancreas and SHOXB in the foetal kidney (Rao et al., 1997). SHOX is a pseudoautosomal gene that has been linked to idiopathic short stature and Turner syndrome (Rao et al., 1997; Shanske et al., 1999; Clement-Jones et al., 2000; Blaschke and Rappold, 2000, 2001). In addition, Prx3 is deleted in the similar Leri-Weill dyschondrosteosis syndrome (Belin et al., 1998; Shears et al., 1998; Blaschke and Rappold, 2000, 2001; Huber et al., 2001).

**Craniofacial birth defects**

Many babies are born each year with birth defects. The parents of one out of every 28 babies receive the frightening news that their baby has a birth defect. A birth defect is an abnormality of structure, function or body metabolism (inborn error of body chemistry) present at birth that results in physical or mental disability, or is fatal. A number of well known craniofacial disorders will be discussed below.

**Orofacial clefting**

Orofacial clefting is among the most frequently occurring cranial defect. Cleft lip with or without cleft palate occurs in 1 out of 350 births and cleft palate alone occurs less frequently. Cleft lip may be unilateral or bilateral. Unilateral cleft lip results from failure of one maxillary prominence to merge with the nasal processes, while bilateral cleft lips are due to the failure of both maxillary processes to fuse (see Fig. 1.7). Cleft palate, with or without cleft lip, is caused by a failure of the palatal shelves to meet and fuse with each other, with the nasal septum, and/or with the posterior margin of the primary palate (see Fig. 1.7). The causes of orofacial clefting are not fully understood. Studies suggest that a number of genes, as well as environmental factors, such as drugs (including antiseizure drugs), infections, maternal illnesses, maternal alcohol use and, possibly, deficiency of B vitamin folic acid may be involved (Thorogood, 1997).
Neural tube defects

Neural tube defects (NTDs) occur in 1 of 1000 life births (Edmonds and James, 1990). Spina bifida, meningocele and meningomyelocele are caused by incomplete closure of the neural tube at spinal cord levels, whereas anencephaly and encephalocele are caused by neural tube closure defects at the more anterior brain levels (Campbell et al., 1986; Copp et al., 1990). The causes of the neural tube closure defects are in most cases unknown, but results from many studies show that there is a link between neural tube closure defects and decreased folate levels. Therefore treatment of pregnant mothers with folic acid at about 29 days of pregnancy strongly reduces the risk on neural tube closure defects (Smithells et al., 1989). *Caret* mutant mice have acrania and meroonencephaly at birth, but prenatal treatment with folic acid strongly reduces the incidence of neural tube closure defects (Zhao et al., 1996).

Craniosynostosis

Craniosynostosis is a birth defect that occurs in 1 out of 1600 and often occurs as a part of other malformation syndromes of which the best known are Apert, Pfeiffer, Saethre Chotzen and Crouzon syndromes. It is characterized by premature closing of one or more cranial sutures and it leads to malformation of the cranial cavity and other craniofacial features (Thorogood, 1997). Dominant missense mutations in the gene encoding fibroblast growth factor receptors (FGFRs) 1-3 cause the development of many craniosynostosis syndromes (Muenke and Schell, 1995; Wilkie et al., 1995; Naski and Ornitz, 1998). Recently a mouse mutant was generated
with gain-of-function mutations in the \( F_{gfr2} \) gene. This mouse has a phenotype with strong parallels to some Apert’s and Pfeiffer’s syndrome patients (Hajhosseini et al., 2001).

**Holoprosencephaly**

Holoprosencephaly (HPE) is a disorder with an incidence of 1 of 16,000 life births and 1 of 250 induced abortions that results from a failure of the embryonic forebrain to separate into cerebral and lateral hemispheres, coupled with an abnormal development of the frontonasal process (Cohen, 1989a, 1989b, 1992). A progressive range of craniofacial malformations can be recognized. A mild phenotype involves absence of the intermaxillary process resulting in midfacial clefting. Further deficiency, coupled with a narrower midbrain and a suspected failure of midline definition of the bilateral olfactory placodes produces faces with reduced nasal structures, sometimes manifest as a small nose with a single nostril (cebocephaly). Even further reduction results in complete absence of nose and olfactory structures, with eyes developing much closer to the midline (hypotelorism). In most severe cases the defects result in a single eye present in the midline (cyclopia; Thorogood, 1997). Mutations in \( SHH \) were shown to cause HPE, making it an important candidate gene for the etiology of the syndrome (Belloni et al., 1996; Roessler et al., 1996).

**Treacher-Collins syndrome**

Treacher-Collins (TCS) is a rare autosomal dominant disorder that occurs in 1 of 50,000 live births (Gorlin et al., 1990). It affects the entire face: the lower jaw is underdeveloped (microagnathia), the palatine is cleft and the external ears are malformed, which often goes together with conductive deafness due to absent or dysmorphic ossicles (Thorogood, 1997). Mutations in the \( TCOF1 \) gene, mostly leading to premature stop codons, cause development of TCS (Dixon et al., 1997; Wise et al., 1997). \( Tcof1 \) haploinsufficiency in mice causes massive increase in the levels of apoptosis in the prefusion neural folds, which likely underlies the development of the craniofacial malformations (Dixon et al., 2000).

**DiGeorge syndrome**

DiGeorge syndrome (DGS) occurs with an estimated frequency of 1 in 4000 live births (Emanuel et al., 1999). Patients have the craniofacial defects as described for Treacher-Collins syndrome, but additionally both the thymus and parathyroids are either absent or reduced and
there are cardiovascular anomalies including persistent truncus arteriosus and dysmorphic aortic vessels. The parathyroid and thymic anomalies are likely consequences of phenotypes in third and fourth branchial pouches associated with defects in neural crest development and arch morphogenesis. The cardiovascular problems are thought to result from disruption of the cardiac neural crest, which emerges from the posterior hindbrain (Thorogood, 1997). DGS is usually associated with deletions of chromosome 22q11 (Scambler, 2000). Recent studies implicate the transcription factor TBX1 as a key candidate gene for the malformations seen in DGS (Jerome and Papaioannou, 2001; Lindsay et al., 2001). However, other genes within a closely linked region of 22q11, like CRKL, might affect the same developmental pathway (Guris et al., 2001).

**Aim of the Ph. D. project and this thesis**

At the start of this project our group had just begun to recognize that the aristaless-related genes have similar functions during embryonic development. We had cloned mouse Prx1 and Prx2, studied their expression patterns during development and suggested the current nomenclature (Leussink et al., 1995). The phenotype of the Prx1 mutant mouse had also been published (Martin et al., 1995). Derk ten Berge had generated the Prx2 mutant mouse and analysed the mouse Alx3 gene during his Ph. D. project (ten Berge et al., 1998a,b). In the Alx3 expression paper we report the existence of a group of aristaless-related genes. The same year the phenotype of the Cart1 mutant mice was published. The description of the Alx4 mutant mouse phenotype followed soon after (Zhao et al., 1996; Qu et al., 1997). The aim of my project was to generate Alx3 mutant mice and to study its function and the functions of the highly related Alx4 and Cart1 genes during craniofacial development. In Chapter 2 the expression patterns of Alx3, Alx4, Cart1 and Prx1-3 in the craniofacial primordia and the outgrowing limbs are described. I suggest that a further categorization of group I aristaless-related genes in three subgroups could be made based upon protein structure, expression patterns and functional data. In Chapter 3, the generation of the Alx3 mutant mouse, the analysis of the Alx3/Alx4 double mutant embryos, the etiology of the phenotype and a probable cellular mechanism leading to the phenotype are described. Chapter 4 describes the skeletal phenotype of Alx3/Cart1 double and Alx3/Alx4/Cart1 triple mutant mice.