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Chapter 1

Short outline of heart development

The vertebrate heart is the first organ to form and function during development. The cells that contribute to the heart are found prior to gastrulation within the posterior half of the epiblast (Rawles, 1943; Hatada and Stern, 1994; Parameswaran and Tam, 1995) and are among the first mesodermal cells to gastrulate through the rostral portion of the primitive streak (Garcia-Martinez and Schoenwolf, 1993; Schoenwolf and Garcia-Martinez, 1995). These cardiac progenitors migrate to an anterior lateral position within the visceral mesoderm where they condense to form the bilateral heart primordia or primary heart fields (DeHaan, 1965). The cardiogenic mesoderm becomes a true epithelium and is separated from the dorsal somatic lateral plate mesoderm by the formation of the coelom (Linask, 1992). From the cardiogenic mesoderm not only the cardiomyocytes differentiate but also the endocardial cells that populate the space in-between the endodermal and mesodermal layer (Linask and Lash, 1993; Wunsch et al., 1994; Sugii and Markwald, 1996).

Due to folding of the embryo the bilateral mesodermal heart fields come together ventrally of the embryo and fuse in the midline to form the cardiac tube (De Jong et al., 1997; Rosenquist and DeHaan, 1966). This heart tube consists of two concentric layers of cells, the myocardial outer layer separated by cardiac jelly from the endocardial inner layer (De Jong et al., 1997). As development proceeds, myocardium is added continuously to the posterior and anterior side of the elongating tube (De La Cruz and Sanchez-Gomez, 1998; Viragh and Challice, 1973; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The dorsal mesocardium ruptures and the heart tube loops to the right. During looping atrial and ventricular myocardium is formed by local differentiation and proliferation of myocardium at the outer curvature (Christoffels et al., 2000; Moorman et al., 2000). The myocardium of the inflow tract, atrioventricular canal, inner curvature and outflow tract escapes this differentiation program until later in development, by local repression of differentiation (Habets et al., 2002). In the atrioventricular canal, outflow tract and inner curvature, the cardiac jelly expands and a subset of endocardial cells undergoes an epithelial-to-mesenchymal transformation forming the mesenchyme of the endocardial cushions, which are the precursors of the valves and septa (Mjaatvedt et al., 1999; Wessels et al., 1996). The endocardial cushions are subsequently populated by mesenchyme derived from the neural crest and epicardium. Cardiac neural crest-derived mesenchyme contributes to the mesenchyme of the outflow tract (Kirby et al., 1983; Kirby and Waldo, 1995; Waldo et al., 1998) and epicardium-derived mesenchyme to the mesenchyme of the atrioventricular canal (Manner et al., 1999; Gittenberger-de Groot et al., 1998). During subsequent development the mesenchymal components of the cushions fuse and become largely muscularized (De Jong et al., 1997; Mjaatvedt et al., 1999; Moorman et al., 2000; Van den Hoff et al., 1999, 2001), in addition, the walls of the pulmonary and caval veins will become myocardial (Van den Hoff et al., 2001).
Myofibrillogenesis

The expression of muscle-specific contractile-protein in chicken is first detected at Hamburger and Hamilton (HH) stage 7 (Hamburger and Hamilton, 1951), which is about 10 hours before spontaneous beating begins (HH10; Han et al., 1992). In mouse, myosin (Lyons et al., 1990) and cardiac actin (Sassoon et al., 1988) are first observed in the primitive heart tube of embryonic day (E) 7.5-8.0, which contracts spontaneously at E8.5 (DeRuiter et al., 1992). The assembly of these contractile proteins into a functional unit, the sarcomere (Fig. 1), must therefore be a rapid and well-coordinated process in early cardiogenesis. The assembly of contractile proteins into myofibrils requires coordinate synthesis of the constituent proteins, the polymerization of actin and myosin and many associated proteins into thin and thick filaments, respectively, and the association of the two filament systems into highly organized sarcomeres.

Immunolocalization studies in the chicken heart have provided a comprehensive description of myofibril assembly in the developing myocyte (Tokuyasu and Maher, 1987a,b; Ehler et al., 1999; reviewed in Gregorio and Antin, 2000). At the earliest stages

![Image of sarcomere](image)

**Figure 1.** Major components of a cardiac muscle sarcomere (Adapted from Gregorio and Antin, 2000). Assembled sarcomeres consist of parallel arrays of ~1.0 mm-long thin filaments and 1.6 mm-long thick filaments. The Z line (Z disc) is part of the sarcomere that separates one sarcomere from the next. Actin filaments are attached with associated proteins to the Z disc and extend toward the middle of the sarcomere where they overlap with myosin filaments. The M line is the midline of a sarcomere. Titin forms a network of fibers extending from the myosin filament to the Z disc. Tropomyosin molecules form two polymers per thin filament; the polymers stabilize the thin filaments. Each tropomyosin molecule binds one troponin complex (composed of troponins T, I and C) and together they mediate the calcium regulation of muscle contraction. α-Actinin is an actin-crosslinking protein. Actin filament capping protein CapZ and tropomodulin inhibit the elongation and depolymerization of the filaments. Other myosin-binding-proteins (MyBP) like MyBP-H, M protein and myomesin are situated in the same region as MyBP-C.
of myocyte differentiation in chicken (HH8, 26-29h), all sarcomeric proteins studied have accumulated diffusely within the cytoplasm, except for titin, which is detected in a punctate pattern that probably represents aggregates of several titin molecules. Within a few hours, titin dots appear along the plasma membrane at sites of α-actinin localization, forming nascent Z bodies (i.e. precursor Z lines). Filamentous actin accumulates near the cell membrane and associates with the Z bodies, forming 1-Z-1 complexes. Myosin also appears first diffusely in the cytoplasm. Slightly later, myosin filaments that are not associated with 1-Z-1 complexes are observed in the cytoplasm (Fig. 2a). At stage HH9-10, myosin thick filaments organize into a sarcomeric arrangement together with 1-Z-1 structures (Fig. 2b). The assembly of the myosin-binding protein myomesin correlates with this event, suggesting that this molecule might anchor thick filaments to thin filaments, analogous to the crosslinking role of α-actinin within Z-lines (Ehler et al., 1999). At this stage, the heart fields have started to fuse and the beginning of beating becomes apparent. Myosin thick filaments are aligned in their mature sarcomeric pattern before the actin thin filaments are organized into their mature pattern. Smooth muscle actin and skeletal actin participate in early formation of striated thin filaments and precedes the expression of cardiac actin (Ruzicka and Schwartz, 1988; Sugi and Lough, 1992). At HH11, when actin thin filaments attain their mature pattern, cardiac actin becomes the predominant myofibrillar actin isoform. The alignment and stabilization of myofibrils is the last step of myofibrillogenesis (Fig. 2c).

![Figure 2. Model of the stages of cardiac-myocyte-sarcomere assembly in the chicken embryo (Adapted from Gregorio and Antin, 2000). a) At HH8 (26-29h) 1-Z-1 complexes composed of Z bodies containing α-actinin and titin linked to actin filaments are primarily associated with the cell membrane. Actin filaments are of variable lengths. Full-length myosin thick filaments are scattered diffusely in the cytoplasm, whereas some are associated with 1-Z-1 complexes. b) At HH9-10 (29-38h), myosin thick filaments are organized together with 1-Z-1 structures, forming immature sarcomeres. c) At HH11 (40-45h), thin filaments have obtained their mature lengths. Individual myofibrils are aligned and continuous between neighbouring cells at the intercalated disks.](image-url)
Transcriptional regulators involved in cardiac development

The cardiac program is initiated in the splanchnic mesoderm by inductive signals from overlying tissues (see later sections). These inductive signals activate expression of a specific subset of transcription factors within the cell to coordinate a complex array of gene expression, responsible for cardiac contractility and morphogenesis. The transcription factor Nkx2.5 is among the earliest transcription factors expressed in the cardiac mesoderm (Lints et al., 1993; Schultheiss et al., 1995). Together with the transcription factor GATA4, Nkx2.5 activates cardiac gene expression (Lee et al., 1998; Durocher et al., 1997). In addition, Nkx2.5 and GATA factors appear to cross-regulate one another's expression (Lien et al., 1999; Molkentin et al., 2000). Slightly later in development, other cardiac transcription factors like Mef2c, d- and eHand, and Tbx5 are expressed in cardiac mesoderm. Although all mentioned transcription factors are highly enriched in cardiac mesoderm and, subsequently, in the linear heart tube, no transcription factor has been found to be essential for myocardial differentiation in mice (Kuo et al., 1997; Molkentin et al., 1997; Lin et al., 1997; Srivastava et al., 1997; Firulli et al., 1998; Riley et al., 1998; Bruneau et al., 2001). Functional redundancy with closely related transcription factors might explain this phenomenon. In addition, no transcription factor is exclusively expressed in cardiac muscle cells or is able to induce full cardiac differentiation ectopically. These findings indicate that the master cardiac-specific transcription factor is still not yet identified or, more likely, that a combination of transcription factors is required for the initiation of the cardiac program.

In Nkx2.5 knockout mice the development of the heart stops at the looping stage (Lyons et al., 1995; Tanaka et al., 1999). The atrial and ventricular chambers fail to expand. In addition, the right ventricle, the endocardial cushions and the ventricular trabecules do not form, suggesting a general inhibition of cardiogenesis after the establishment of the tubular looping heart. Furthermore, heterozygous mutations in human Nkx2.5 were identified in patients with septal defects and atrioventricular conduction defects, suggesting that Nkx2.5 is also important in the later stages of heart development (Schott et al., 1998; Benson et al., 1999). In GATA4 deficient mice, the cardiac mesoderm fails to fuse at the ventral midline to form the linear heart tube, which is probably due to a defect in the folding of the embryo (Kuo et al., 1997; Molkentin et al., 1997). Experiments in *Xenopus* suggest that GATA4 is required for formation of the posterior heart, which is consistent with its expression pattern (Jiang et al., 1998, 1999; Heikinheimo et al., 1994). Functional disruption of Tbx5 caused severe hypoplasia of posterior heart structures in the linear heart tube in mice, showing that also Tbx5 is required for the formation of the posterior heart (Bruneau et al., 2001). Tbx5 has been implicated in controlling cell growth and mutations in this gene are associated with defects observed in the Holt-Oram syndrome (Hatcher et al., 2001; Terrett et al., 1994).
Figure 3. Graphic representations of early stage (HH5-6) chicken embryo. a) The "classic" and the actual heart field. b1) Cartoon indicating the several regions used in experimental biology for the study of cardiomyogenesis. Some of those regions overlap with the presumably actual heart field. b2) Diagram displaying the in vitro differentiation experiments in which the indicated regions are cultured in presence of indicated factors and assessed for full cardiac differentiation. c1) Cartoon indicating the endogenous expression patterns of bone morphogenetic protein (BMP) 2 and fibroblast growth factor (FGF) 8 (Alsan and Schultheiss, 2002[7060]). c2). Diagram displaying the in vivo experiments in the regions depicted in figure c indicating that both BMP and FGF are required for the induction of cardiac gene expression and that BMP functions upstream of FGF. d) Model for the induction of cardiogenesis in explants of anterior and posterior lateral mesoderm by BMP and FGF. In the anterior mesoderm (except for the most lateral mesoderm) BMP can induce full cardiac differentiation by inducing FGF expression. In posterior mesoderm, BMP cannot induce FGF expression and, therefore, no full cardiac differentiation. Addition of FGF is, therefore, required to induce full cardiac differentiation in posterior mesoderm by BMP.
Haplo-insufficiency of Tbx5 in man and mouse causes relatively mild cardiac defects, which include defects in the septa and deficient conduction system (Li et al., 2000; Basson et al., 1999; Bruneau et al., 2001). Mice deficient for Met2e or dHand show malformed anterior heart structures, indicating that they are required for formation of the anterior heart (Lin et al., 1997; Srivastava et al., 1997). Because dHand and Met2e deficient mice exhibit similar phenotypic defects and dHand is down-regulated in Met2e mutant mice, dHand has been implicated to cooperate with Met2e in formation of the anterior region of the heart (Olson and Black, 1999).

The transcription factors Tbx2, -3, -5, and Irx4 are implicated in compartment-specific gene regulation. Tbx2 was found to repress chamber-specific gene expression in the primary heart tube, whereas Tbx5 stimulates the chamber-specific program of gene expression (Habets et al., 2002). Irx4 is suggested to regulate ventricular versus atrial gene expression by stimulating ventricle-specific gene expression and down-regulates atrium-specific gene expression (Bao et al., 1999; Bruneau et al., 2001).

Although not expressed in heart, the transcription factor Mespl is of interest for cardiac development. Lineage analysis showed that a lineage of cells that have expressed Mespl exclusively gives rise to the myocardium, endocardium, (pro)epicardium and their derivatives, and to the endothelial cells of the vascular system (Saga et al., 2000). Because Mespl is expressed at E6.5-7.0 (Saga et al., 1996), Mespl may represent the earliest molecular marker expressed in heart precursor cells.

### Formation and elongation of the linear heart tube

The tubular heart is formed by migration and fusion of the two heart-forming regions at the ventral midline (chicken HH9-10; Stalsberg and De Haan, 1969; Rosenquist and De Haan, 1966; mouse E7.5-8; DeRuiter et al., 1992). As development proceeds, myocardium is added to the posterior side of the tube forming the inflow region (Stalsberg and De Haan, 1969; De la Cruz and Markwald et al., 1998; Viragh and Challice, 1973) and to the anterior side, which will contribute the outflow tract in chicken (De la Cruz and Markwald et al., 1998; Mjaatvedt et al., 2001; Waldo et al., 2001) and the outflow tract and possibly the embryonic right ventricle in mouse (Kelly et al., 2001; Viragh and Challice, 1973). Fate maps of different stages have been prepared to identify cells that will eventually contribute to the heart (Rawles, 1943; DeHaan, 1963; Stalsberg and DeHaan, 1969; Rosenquist and DeHaan, 1966; Ehrman and Yutzey, 1999; Redkar et al., 2001; Garcia-Martinez and Schoenwolf, 1993). In these studies the (classic) heart-forming fields of HH15 embryos are mapped in the anterior lateral mesoderm (Fig. 3a). A recent lineage study by Redkar and co-workers (Redkar et al., 2001) showed that the heart-forming region of HH15 embryos extends more posterior than the heart-forming region defined by Ehrman and co-workers (Ehrman and Yutzey, 1999). This discrepancy may result from
the difference in stage of analysis between both studies, since Redkar and co-workers analyzed the fate of the cells at HH12, whereas Ehrman and co-workers did this at HH10. These findings indicate that between HH15 and HH12 ongoing recruitment of mesodermal cells to the cardiogenic lineage has taken place.

The actual heart-forming region might even be larger (Fig. 3a), because still myocardium is added to the tubular heart at the anterior and posterior pole of the heart after HH12 (De la Cruz and Sanchez-Gomez, 1998; Kelly and Buckingham, 2002; Van den Hoff et al., 1999 and 2001). Cell-marking experiments in living embryos showed that the outflow tract myocardium is added to the chicken heart between stage HH19 and HH24 (De la Cruz et al., 1977; Waldo et al., 2001; Van den Hoff et al., 2002), and to the mouse heart between E8 and E11 (Kelly et al., 2001). The addition to the posterior side has always been considered to be a continuous addition of myocardium from the primary heart fields, whereas the myocardium added to the anterior side has been suggested to be derived from a distinct heart-forming field. This splanchnic mesoderm cranial to the heart tube is referred to as the anterior or secondary heart field (De la Cruz and Markwald et al., 1998; Mjaatvedt et al., 2001; Waldo et al., 2001; Kelly et al., 2001). The name “secondary heart field” (Waldo et al., 2001; Yutzey and Kirby, 2002) is in our opinion misleading, as we will outline below. The distribution of transcripts from the FGF10 enhancer trap transgene, which might be activated in myocardial precursor cells of the anterior heart-forming field, suggests that the myocardial precursor cells of the anterior heart-forming field are lying medial to the cells of the classic heart fields in the mesoderm in 7.5 day mouse embryo (Fig. 3a and 4; Kelly et al., 2001). These observations indicate that the initial heart field is much broader, comprising laterally the ‘classic’ heart field, and

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**Figure 4.** Heart development between E7.5 and E9 of mouse development showing the anterior heart forming field (AHF; dark grey) in relation to the classic heart-forming fields, i.e. posterior heart forming field (PHF; light grey; Adapted from Kelly et al., 2001). The boundary between the contribution of the AHF and of the paired heart-forming fields to the embryonic heart may lie in the interventricular region. Transverse sections corresponding to the horizontal lines in the E8.25 and E9 diagrams are shown below. Between the diagrams of E8.25 and E9 a sagittal section is shown in which the dorsal mesocardium has ruptured. AP, arterial pole; DM, dorsal mesocardium; LV, embryonic left ventricle; RV, embryonic right ventricle; VP, venous pole.
medially the precursors of the ‘novel’ anterior heart-forming field. Fate-map studies in chicken embryos had not identified these precursors as the New culture system used in these studies does not allow propagation of the embryos beyond HH12, whereas the outflow tract myocardium is added up to stage 24. When the heart tube forms and the foregut invagination occurs, the cells contributing to the outflow tract are displaced dorsally and anteriorly relative to the heart tube (Fig. 4). Rupture of the dorsal mesocardium isolates these cells behind the looping heart tube, although they remain contiguous with the arterial pole (Kelly and Buckingham, 2002) and posterior pole, creating an ‘anterior heart-forming field’. Reminiscent to the anterior heart-forming field, the area contributing to the posterior side of the heart tube could be called ‘posterior heart-forming field’ (Van den Hoff et al., 2001). In both anterior and posterior heart-forming fields of the chicken embryo the cardiac transcription factors Nkx2.5 and GATA4 are expressed (Waldo et al., 2001; in mouse embryo, GATA4 expression is found in the posterior heart-forming field (Heikinheimo et al., 1994).

Taken together we think that accretion of myocardium at both the venous and arterial pole of the heart is a continuation of the formation of myocardium of the initial linear heart tube. In this view, the outflow tract myocardium is not added from a special secondary heart field, but from a continuous single heart field, which comprises both the anterior and posterior heart-forming fields (Fig. 3a). This view does not imply that the anterior and posterior heart-forming fields are similar. Most likely they become different imposed by different positional cues. The notion that the anterior heart field is located medial to the primary heart fields is supported by the observation that both anterior medial and anterior lateral mesendoderm are possessing equivalent properties to induce cardiomyogenesis in posterior primitive streak explants of chicken (Schultheiss et al., 1995) and have both the potency to form cardiac muscle in culture (Schultheiss et al., 1997).

**Myocardial differentiation**

**Experimental approaches**

To study the commitment to the myocardial lineage and the (molecular) mechanisms underlying the differentiation of myocardial cells, two culture systems are generally used, being 1) Explant culture assay, and 2) Whole embryo culture.

*Explant culture assay.* In explant assays, the ability of explants to display myocardial differentiation is determined under various culture conditions. In explant cultures which support cardiogenesis, non-cardiac cells migrate from the explant while within the central region cardiac myoblasts proliferate, form multilayers, and commence rhythmic contractions (Sugi et al., 1993). Explants studies have several limitations and pitfalls.
Basis | medium | serum | refs |
--- | --- | --- | --- |
fibronectin-coated chamber slides | 75% DMEM; 25% McCoy's | - | a |
fibronectin-coated chamber slides | M199 | - | b |
fibronectin-coated chamber slides | DMEM | - | c |
tibronectin-coated multi-well dish | DMEM | 20% FCS and 1% CEE | d |
collagen I coated chamber slides | M199 | - | e |
collagen gel type 1 | CEM | 10% FCS, 5% CEE | f |
floating filter rafts | CEM | 10% FCS, 5% CEE | g |
floating filter rafts | F10C | 2% CEE | h |
gelatin-coated multi-wells | De Haan's medium | 2% HS and 4% FCS | i |
gelatin-coated multi-wells | DMEM | 20% FCS and 1% CEE | j |


Firstly, the culture conditions used vary extensively among the different laboratories (Table 1). Culturing the same region of an embryo under different conditions was observed to result in apparent contradictory observations (see footnote1). Secondly, limitations imposed by the requirement for meticulous microdissection preclude the precise exclusion of cells from the germ layers (Sugi and Lough, 1994). Note that a chicken embryo of HH5 is only 2.5 mm long. Consistent separation of HH5 anterior lateral endoderm from overlying cardiogenic mesoderm was not possible, resulting in the occasional appearance of cardiac myocytes in explants of isolated endoderm (Yatskievych et al., 1997). Reproducible and precise isolation of the region of interest is of great importance, since contaminations can have significant influence on the results. Although it would be preferable to culture solely mesoderm to study cardiomyogenesis, mesoderm is generally cultured in combination with the underlying endoderm (mesendoderm),

1 DMEM/McCoy's medium supports mesodermal survival, M199 does not. Gannon and Bader (1995) cultured mesoderm in the presence of ectoderm since this was required for the mesoderm to survive. Sugi and Lough (1994) also reported that isolated mesodermal explants are not able to survive alone in culture. The mesodermal cultures of Antin and co-workers (1994), however, did survive alone in culture. The difference in survival of mesodermal explants as observed between research groups might be due to different culture conditions (table 1). Antin et al., (1994) cultured the mesodermal explants on fibronectin-coated chamber slides in DMEM/McCoy's medium, whereas Gannon and Bader (1995) cultured the mesodermal explants on 0.01% collagen I coated chamber slides in M199 medium. Since, mesodermal explants that were cultured on fibronectin-coated chamber slides in M199 medium, did not survive (Sugi and Lough; 1994), it can be concluded that in this case the medium is the limiting factor in the survival of the mesodermal explants and that DMEM/McCoy's medium supports survival of mesodermal explants better than M199 medium.
because of the difficulty of removing the endoderm completely without damaging the mesoderm.

**Whole embryo culture.** To manipulate the chicken embryo at the time of cardiac differentiation, the embryos need to be cultured in a so-called New culture (New, 1955; Chapman et al., 2001). In a New culture, very early stage embryos that are isolated along with the surrounding egg membranes, are cultured ventral-side up on a substrate. The embryos need to be cultured up side down to allow manipulation of the heart-forming regions. This culture system allows placing of beads soaked in recombinant purified growth factors (Schultheiss et al., 1997). Cell lines expressing factors of interest (Andree et al., 1998) can be placed adjacent to the (pre) cardiac region, and cell layers or parts of the developing embryo can be removed (Alsan and Schultheiss, 2002; Gannon and Bader, 1995). In New cultures, the embryos can be cultured till about HH12. After the incubation period the embryos are fixed and analyzed by whole mount in situ hybridization, or gene expression is assessed by semi-quantitative RT-PCR.

During the acquisition of the myocardial phenotype, general stages can be distinguished, being (i) the expression of cardiac transcription factors, (ii) the expression of sarcomeric proteins, (iii) the assembly of the contractile proteins, and (iv) beating activity. Although differentiation of myocardial cells can be determined by the presence of markers of these stages, beating identifies full cardiac differentiation. To determine whether cardiogenesis is initiated and till which stage it has proceeded, it is important to analyze the expression of several markers representative of the four stages. Firstly, because the absence of one cardiac marker or beating does not necessarily imply that myocardial differentiation has not occurred. Secondly, because the commonly used cardiac markers are not exclusively expressed in the heart during development. For example cardiac actin, myosin light chain 1A (MLC1A) or ß myosin heavy chain (MHC) are also expressed in developing skeletal muscle (Buckingham et al., 1992). In addition, expression of the so-called cardiac-specific transcription factors is not confined to the developing heart. Thus, examining only one or a few cardiac markers might lead to (1) a failure to detect cardiomyogenesis or (2) to over-interpretation as also non-cardiac tissues are identified.

**Commitment of cells to the myocardial lineage**

Already early during development cells become committed to the cardiac lineage. The level of commitment of mesodermal cells to the cardiac lineage was investigated for several regions of the embryo by embryological experiments. Two levels of commitment have been defined, being specification and determination. Specification denotes the acquisition of a level of developmental commitment that is sufficient for tissue formation when the cells are cultured in isolation. Determination denotes the acquisition of a level
of developmental commitment that is not only sufficient for tissue formation when the cells are cultured in isolation, but in addition, the commitment is also irreversible regardless of the environment; the determined tissue will continue to develop autonomously even after it is moved to any other region of the embryo (Slack, 1983).

**When do myocardial cells become specified?**

Cells that will form the heart are found prior to gastrulation within the lateral posterior epiblast and become progressively localized toward the primitive streak as gastrulation commences (Hatada and Stern, 1994; Lawson et al., 1991). Chicken epiblast cells isolated from the heart-forming primitive streak regions of embryos of HH3 formed cardiac muscle cells in culture, indicating that at HH3 myocardial cells are specified (Yatskievych et al. 1997). Prior to HH3, cardiac muscle cell formation in epiblast culture is dependent upon a signal from the hypoblast (Yatskievych et al., 1997). Activin A, transforming growth factor (TGF) β1 or fibroblast growth factor (FGF) 4 were found to substitute for hypoblast. However, neither of these factors was able to convert noncardiogenic mesoderm isolated from HH5 chicken embryos into cardiomyocytes (Ladd et al., 1998). These findings indicate that the role of activin A, TGFβ1 or FGF4 in this process might be indirect, possibly by promoting the formation of precardiac mesoderm competent to respond to heart-inducing signals. In agreement with this hypothesis, activin A (Asashima et al., 1990; van den Fienden-Van Raaij et al., 1990), TGFβ1 in combination with FGF2 (Kimelman and Kirschner, 1987), and FGF4 (Paterno et al., 1989) have all been found to be capable of inducing mesoderm formation. In addition, the activin receptors, ActRIIA and ActRIIB, and the FGF receptor 1 (FGFR1) have been found to be required for mesoderm formation (Song et al., 1999; Ciruna et al., 1997). Taken together, this might indicate that in epiblast cultures prior to the formation of cardiac muscle, cells of the germlayers are formed from which, subsequently, the mesodermal cells are being specified resulting in the differentiation into cardiac muscle cells. This might indicate that epiblast cells are not specified to the cardiac lineage. To test the hypothesis that epiblast cells are specified to the cardiac lineage, epiblast cells have to be used that form only mesodermal and no endo- or ectodermal cells in culture. Because this is not possible at present, specification of myocardial cells can only be studied in mesodermal explants.

With respect to cardiomyogenesis in mesoderm, specification reflects the ability of mesoderm from the heart forming regions to form cardiomyocytes when cultured in a defined medium without the adjacent endo- and ectoderm (see footnote²). Antin and co-

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² **Study of the specification of cardiac mesoderm.** Several studies reported that specification of heart precursors is well underway by midgastrulation (HH4; Gonzalez-Sanchez and Bader, 1990; Antin et al., 1994; Gannon and Bader, 1995). This was determined by defining the ability of explants from specific regions of the early embryo to differentiate into beating cardiac myocytes. Gonzalez-Sanchez and Bader (1990), however, cultured the mesoderm in the presence of serum
workers (1994) showed that anterior lateral mesodermal explants (Fig. 3b) of the heart forming regions taken from the first stage (HH4+) at which mesoderm can be isolated, are able to differentiate into cardiac muscle cells in minimal medium as determined by the expression of MHC and the presence of beating. This indicates that specification of myocardial cells is well underway by midgastrulation and thus seems to occur prior to or during early gastrulation. In agreement, heterotopic transplantation studies performed on mouse embryos are consistent with the findings in chicken, indicating that specification may begin prior or during early gastrulation (Tam et al., 1997). Specification of myocardial cells may continue at least till stage 24 in chicken and E11 in mouse, because till that stage myocardium is still added to the heart tube (as discussed above). In addition, because septa in the embryonic heart and the caval and pulmonary veins will become myocardial, specification may even proceed after those stages.

The mesoderm of the posterior region of the chicken embryo is considered to be non-precardiac (Rawles, 1943; Stalsberg and DeHaan, 1969; Ehrman and Yutzey, 1999). In agreement, explanted posterior mesendoderm from HH5 and 6 embryos does not develop into beating cardiac tissue (Ladd et al., 1998; Sugi and Lough, 1994). However, in some cultures cells are present that express low levels of MHC (Eisenberg and Eisenberg, 1999). In addition, in explanted posterior mesendoderm cultures from HH4 embryo, beating cardiac tissue is formed (Eisenberg and Eisenberg, 1999). These findings may indicate that mesodermal cells possess a degree of plasticity that allows them to change their fate upon changing environment conditions. Possibly, posterior mesoderm possesses a higher degree of plasticity at HH4 than at HH5. However, since the heart-forming field extends more in posterior direction than first thought (as discussed above; Fig. 3a), the presence of cardiomyocytes in posterior mesendoderm cultures isolated at HH4-6 may be explained by the presence of precardiac cells from the most posterior part of the heart-forming field (Fig. 3b). To minimize the chance that posterior mesoderm explants comprise precardiac cells, the mesoderm should be isolated from the most posterior region of the embryo (Fig. 3b).

Reminiscent to the posterior mesoderm, the anterior medial mesoderm was initially also considered to be non-precardiac. Since the heart-forming field extends also medially more than initially thought, anterior medial mesoderm explants may easily be contaminated with precardiac mesoderm of the heart-forming field. This is supported by the observations that isolated anterior medial mesendoderm of HH5 already expresses rather than in a defined medium. The serum might have substituted for the specifying signal from absent tissues. Gannon and Bader (1995) cultured the mesoderm not completely devoid of other cell layers, but in presence of ectoderm (see technical note 1). Sofar, only Antin and co-workers (1994) cultured pure mesoderm in a defined serum-free medium and observed contractility. No markers, however, were used to show that the isolated mesoderm was not contaminated with endoderm. Anterior endoderm explants of any size were found to be capable of causing cardiogenesis in precardiac mesoderm (Sugi and Lough, 1994).
GATAs, Me2a, and Nkx2.8 mRNA based on RT-PCR analysis. During culture of the anterior medial mesendoderm these transcripts disappeared, whereas low levels of Nkx2.5 mRNA were detected after 48 hours of culture (Schlange et al., 2000; Schultheiss et al., 1997).

**When do myocardial cells become determined?**

After initial specification, cells continue to become specified to the cardiac lineage and specified cells become stronger committed to the cardiac lineage. Several studies have suggested at least two levels of commitment. Studies in the axolotl suggested that first the expression of contractile proteins is induced, and a second signal induces the assembly of the contractile proteins in myofibrils (Smith and Armstrong, 1990). A study of Antin et al., (1994) suggested that avian precardiac mesodermal explants undergo a transition to a higher level of commitment around stage 6, since they appear less influenced by their surrounding environment. Other studies showed that avian precardiac tissues become resistant to the chemicals 12-O-tetradecanoylphorbol-13-acetate (TPA) from HH8 onwards (Gonzalez-Sanchez and Bader, 1990) and to bromodeoxyuridine (BrdU) from HH7 onwards (Montgomery et al., 1994), whereas before these stages the chemicals inhibited cardiogenesis in precardiac tissues. These findings suggest that after specification another level of commitment is obtained among HH6-8, which may indicate that the precardiac tissue is determined (Table 2). From HH6 onwards, the phenotypic diversification between anterior and posterior cells is stable (Patwardhan et al., 2000), indicating that cardiomyocytes are determined to their anterior-posterior fate. Transplantation studies to non-cardiac sites of the embryo should be done to define whether these cells are completely determined.

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<th>Commitment of myocardial cells</th>
<th>specified</th>
<th>determined</th>
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Table 2. Myocardial cells are specified at or before HH4. At the stages HH6-8 they become most likely determined to the myocardial lineage upon a signal from the endoderm.
Role of endoderm in the commitment to the myocardial lineage

Precardiac cells have been shown to reside in close proximity to pharyngeal endodermal cells from the time of gastrulation until terminal differentiation (DeHaan, 1965). Although initially precardiac cells are also in contact with ectodermal cells, fully differentiated heart muscle cells were rarely observed in ectoderm-mesoderm explants from HH4+ embryos (Antin et al., 1994; Gannon and Bader, 1995). In contrast, mesoderm explants from HH4+ embryos were able to differentiate into beating cardiac muscle cells (Antin et al., 1994). The presence of endoderm enhanced the rate of myocyte differentiation (Antin et al., 1994). These observations suggest an inhibitory role of ectoderm and a stimulating role of endoderm.

Cells destined to form the heart pass through the primitive streak at HH3 (Garcia-Martinez and Schoenwolf, 1993), approximately coincident with the movement of endodermal cells through the streak. Since precardiac mesoderm is specified by stage 4+, any signaling interaction between endoderm and mesoderm would occur while cells are in close apposition within the streak and prior to formation of a definitive endodermal layer. Techniques are presently not available to separate these two cell types at this stage to determine whether endoderm is required for the specification of the mesoderm.

To get insight in the ability of endoderm to specify cells to the cardiogenic lineage, the ability of endoderm to (re)specify non-cardiac cells to the cardiogenic lineage has been determined. Anterior lateral (mes)endoderm of HH5 was found to induce cells of the posterior primitive streak, which are normally not fated to become heart, to differentiate in cardiomyocytes as determined by the expression of MHC (Fig. 5,a,b; Schultheiss et al., 1995; Marvin et al., 2001). In addition, anterior lateral mesendoderm of HH5 was found to induce cardiomyocytes differentiation in posterior lateral mesoderm (Fig. 5c; Marvin et al., 2001). These findings suggested that factors expressed by the anterior lateral endoderm play an important role in myocardial cell specification in vivo (Schultheiss et al., 1995). The cardiac-promoting activity appeared to be specific to the anterior endoderm, because anterior lateral mesoderm is not able to induce MHC in posterior primitive streak (Fig.5d), and posterior mesendoderm is hardly able to induce MHC or Nkx2.5 in posterior primitive streak (Fig. 5e; Schultheiss et al., 1995). Whether the anterior endoderm expresses all factors necessary to induce cardiogenesis in isolated non-cardiac mesoderm is still unclear. The posterior primitive streak consists of all 3 germlayers and factors from adjacent germlayers might participate in the induction of cardiogenesis by anterior lateral endoderm. In addition, the anterior lateral endoderm has never been shown to induce cardiomyogenesis in serum-free non-precordial mesodermal culture (Fig. 5f). This implies that in addition to the factors provided by the anterior endoderm, an additional factor might be required for myocardial induction.

Removal of anterior endoderm at HH4-6 in vivo abolished the expression of cardiac transcription factors Nkx2.5 and Mef2c in precordial mesoderm at HH 8-9 (Alsan
and Schultheiss, 2002), but not of MHC and cardiac troponin T (cTnT) in the heart tube at HH10-11 (Gannon and Bader, 1995; Antin et al., 2002), indicating that the continuous exposure to endoderm in vivo is not required for maintenance of myocardial differentiation, but is required for the expression of Nkx2.5 and Mef2c. Although the expression of these contractile proteins was not overtly effected, the heart tube did not initiate beating. Chicken embryos in which the endoderm was removed at HH7-9 did not show beating heart tubes, indicating that the endoderm is shortly required at HH7 for the initiation, but not for the subsequent maintenance of terminal differentiation in cardiac mesoderm (Table 2; Gannon and Bader, 1995). In vitro, endoderm is not required for full cardiac differentiation, since pure precardiac mesodermal explants of equivalent stage are able to differentiate into beating cardiomyocytes without endoderm (Antin et al., 1994). Nevertheless, explants comprising mesoderm and endoderm showed an enhanced rate of myocyte differentiation and a shorter delay between expression of myosin heavy chain and the onset of beating. Endoderm, however, is needed for the formation of three-dimensional heart structures in explant cultures (DeHaan, 1964; Lough et al., 1990; Antin et al., 1994), indicating a role for the endoderm in cardiac morphogenesis.

Taken together, endoderm is likely to be required for the initiation but not for the maintenance of both specification and terminal differentiation. In addition, endoderm is

![Diagram of tissue induction](image-url)

**Figure 5.** The ability of several tissues to induce myocardial differentiation in explanted non-cardiac tissue. AL, anterior lateral; PL, posterior lateral; PPS, posterior primitive streak. Refs: a,b,c Schultheiss et al., 1995; b,c Marvin et al., 2001; a Yatskievich et al., 1997.
needed for the maintenance of expression of Nkx2.5 and Mef2c and possibly for cardiac morphogenesis.

**Which growth factors regulate myocardial differentiation?**

The decision whether a mesodermal cell enters the myocardial differentiation pathway and how far that mesodermal cell is able to go into the differentiation pathway, depends on the total set of positive and negative signals that the mesodermal cell will encounter on its way to its final destination.

**BMP and FGF signaling pathways**

Since the only prerequisite for epiblast cells to differentiate into cardiac muscle cells is the placement of those cells in the heart forming fields, all information for heart muscle differentiation is present in the heart forming fields (Tam et al., 1997). Therefore the expression of candidate factors involved in myocardial differentiation in the region of the precardiac mesoderm was examined. Since in *Drosophila*, the cardiac expression of tinman is regulated by the TGFβ family member decapentaplegic (dpp; Frasch, 1995), it was determined whether TGFβ family members also regulate expression of Nkx2.5 and other cardiac genes in vertebrates. Bone morphogenetic proteins (BMPs), which are members of the TGFβ superfamily, are secreted signaling molecules implicated in mesoderm formation, and development and patterning of many different organ systems (reviewed in Hogan, 1996). In situ hybridization showed expression of BMP2, BMP5 and BMP7 expression in the cardiac mesoderm in mice (Zhang and Bradley, 1996; Solloway and Robertson, 1999). In chicken, on the other hand, BMP2 expression is found in the endoderm, and BMP4 and BMP7 expression in the ectoderm flanking the heart forming fields (Fig. 6a and g; Schultheiss et al., 1997). This spatiotemporal pattern of BMP expression suggests a role in early heart development.

Indeed, the BMP antagonist noggin was shown to inhibit cardiogenesis in chicken precardiac mesendoderm of HH4 as determined by the absence of the transcription factors Nkx2.5, GATA4, eHand, and Mef2a, the contractile proteins MHC, α-actinin, and titin, and beating activity (Schultheiss et al., 1997; Nakajima et al., 2002; Schlange et al., 2000). Although noggin did not block the expression of the contractile proteins vMHC, α-actinin, and titin anymore at HH5, the formation of the thick filaments of MHC (A-bands) was inhibited (Nakajima et al., 2002). The formation of I-Z-I components (the Z-disk components being α-actinin, α-actin, and titin) was not inhibited (Nakajima et al., 2002), suggesting that BMP regulates the initial formation of I-Z-I components and A-bands separately in a stage-dependent manner. From HH6 onward, mesendoderm of the
Figure 6. Heart inducing signals in early chick of HH 4-6. a) BMP-2, BMP-4 and BMP-7 are expressed in primitive streak and in the lateral regions of the embryo. b) FGF2, 4, and 8 are expressed in the primitive streak and in region of heart forming fields. c) Wnt3a is expressed in primitive streak. d) Crescent is expressed in the anterior region of the embryo, whereas e) Wnt8c is expressed in posterior region of the embryo. f) Wnt11 is expressed in similar pattern as Nkx2.5. g) Overview of factors influencing the myocardial differentiation in anterior lateral mesoderm. The anterior lateral mesodermal cells are uniformly exposed to a subset of cardiac-inducing signals from the anterior endoderm, ectoderm and mesoderm itself, which supports terminal differentiation and comprises crescent and Wnt-11, which establish high Wnt/β-catenin signaling and low Wnt/β-catenin signaling, and BMPs and FGFs. BMP2, crescent, FGF2, 4 and 8 are expressed by the endoderm, BMP4 and BMP7 by the ectoderm and Wnt11 by the cardiac mesoderm. Expression patterns based on: FGF2 and 4, Karabagli et al., 2002; Parlow et al., 1991; Sugi et al., 1993; FGF4, Shamin and Mason, 1999; FGF8, Alani and Schultheiss, 2002; BMP2, BMP4, BMP7 Schultheiss et al., 1997; Crescent, Wnt3a, Wnt8c, Marvin et al., 1997; Wnt11, Eisenberg et al., 1997; Nkx2.5, Schultheiss et al., 1995.
heart forming regions becomes resistant to the administration of noggin since beating cardiac tissue is formed in these cultures, indicating that BMP is not required anymore for full myocardial differentiation (Schultheiss and Lassar, 1997). In contrast to the above-mentioned genes, GATA4 expression is upregulated from HH5 till HH8 upon addition of noggin (Schlage et al., 2000). Since it has been shown that GATA4 gene is down regulated in presumptive ventricle at HH9 (Kostetskii et al., 1999), the upregulation of GATA4 in the precardiac mesodermal explant culture treated with noggin is attributed to the lack of myocardial differentiation (Schlage et al., 2000).

These in vitro findings are in agreement with the in vivo findings, which show that noggin inhibits at HH4 and HH5 Nkx2.5 and cHand expression, whereas from HH6 the extent of inhibition by noggin gradually diminishes (Table 2; Schlage et al., 2000). Also in vivo noggin inhibited the expression of GATA4 at HH4, but stimulated the expression at HH5-8 (Schlage et al., 2000). In addition, supplementation of noggin at HH4-6 resulted at HH10-11 in the formation of a simple epithelium instead of a tubular heart and no separation of endocardium and myocardium was visible (Schlage et al., 2000). Taken together, these findings show that BMPs have stage-dependent roles in cardiomyogenesis.

Since the anterior endoderm was found to induce cardiomyogenesis in non-cardiac tissue, and expression of BMP2 is the only BMP-family member that is observed in the anterior endoderm in chicken as yet (Schultheiss et al., 1995), BMP2 was the prime candidate to induce cardiomyogenesis. Supplementation of BMP2 in vitro induced ectopic expression of the early cardiac markers Nkx2.5 and GATA4 in anterior medial mesoderm (Fig. 3c), whereas only GATA4 expression was induced in posterior medial mesoderm (Schultheiss et al., 1997; Armand et al., 1998). MHC expression or beating was not observed. Treatment of explanted anterior medial mesoderm or mesoderm of HH6 with BMP2 (or BMP4, but not FGF2 or Activin A) resulted in full cardiac differentiation as assessed by vMHC expression and beating (Fig. 3b2). If anterior medial mesoderm was cultured in the presence of the adjacent neural plate and notochord, BMP2 did only induce Nkx2.5 and Gata4 expression and not MHC (Schultheiss et al., 1997), indicating inhibitory effects of the axial tissues on BMP2-induced cardiogenesis downstream of Nkx2.5 and Gata4. BMP2 did not induce Nkx2.5 in explants of posterior medial mesoderm (Schultheiss et al., 1997), suggesting that induction of full differentiation by BMP2 is restricted to anterior mesoderm (in the presence of endoderm). Although the anterior medial mesoderm is considered as non-precordial, anterior medial mesoderm explants may easily contain precordial mesoderm of the heart-forming field, since the heart-forming field extends more medially than initially thought (Fig. 3b; as discussed above). Therefore, addition of BMP2 may promote the differentiation of already committed precordial myocytes instead of changing the fate of non-precordial cells.

Although full cardiac differentiation as assessed by beating was not observed, BMP2 or BMP4 administration to explants of posterior lateral mesoderm of HH6
resulted in expression of sarcomeric proteins like α-actinin, titin, but not α-actin, indicating that BMP4 and BMP2 can induce myocardial differentiation in presumably non-precardiac posterior mesoderm (Nakajima et al., 2002). In addition, BMP4, but not BMP2, induced myosin expression, although striated myosin pattern (A-bands) was not observed (Ladd et al., 1998; Nakajima et al., 2002). An additional factor appeared to be required to induce terminal differentiation of cardiomyocytes in posterior lateral mesoderm. A combination of BMP2 or BMP4 plus FGF2 or FGF4 appeared to be sufficient to induce full cardiac differentiation in posterior mesoderm of HH6 (Lough et al., 1996; Barron et al., 2000; Ladd et al., 1998), as determined by beating activity and α-actin expression. Thus, in addition to BMPs, FGFs are also required for full cardiac differentiation. This is consistent with their expression pattern in the early embryo. FGF2 and FGF4 have been reported to be expressed during gastrulation in the primitive streak region (Karabagli et al., 2002) and subsequently in anterior lateral endoderm (Fig. 6b; Parlow et al., 1991; Sugii et al., 1993). Since these FGFs have been shown to support the survival and proliferation, and thereby the terminal differentiation of (non)precardiac mesoderm (Zhu et al., 1996; Sugii and Lough, 1995; Lough et al., 1996), it was suggested that FGF is a survival and proliferation factor. In agreement, the FGFRI, which is expressed in precardiac mesoderm of HH6 (Sugii et al., 1995), has been shown to be required for proliferation of precardiac mesoderm (Zhu et al., 1999).

Insulin, insulin-like growth factor II, and activin A are also secreted by the anterior lateral endoderm and stimulate terminal differentiation of precardiac mesoderm (Zhu et al., 1996; Sugii and Lough, 1995; Antin et al., 1996). However, they cannot in combination with BMP2, substitute for FGF2 or FGF4, in inducing full cardiac differentiation in posterior lateral mesoderm, suggesting that the function of FGF in the cardiogenic process is broader than only being a survival or proliferation factor (Barron et al., 2000). This is supported by the observation in chicken that exogenous supplied FGF8, which is expressed in primitive streak and anterior lateral endoderm (Fig. 6b), rescued the expression of the cardiac markers Nkx2.5 and Mef2c in vivo after their down-regulation, which was due to the removal of endoderm (Alsan and Schultheiss, 2002).

Application of BMP2 medial to the heart-forming region in vivo resulted in ectopic expression of FGF8 and Nks2.5, whereas BMP2 expression lateral to the heart-forming region did not result in ectopic expression of FGF8 and Nks2.5 (Fig. 3c; Alsan and Schultheiss, 2002). Application of ectopic FGF8 lateral to the heart-forming region where BMP is present resulted in ectopic expression of cardiac markers Nkx2.5 and Mef2c (Fig. 3c). These findings suggest that both BMP and FGF together are required for cardiac gene expression and that BMP signaling lies upstream of FGF8. In addition, BMP2 seems only to induce cardiac gene expression, if it is able to induce FGF expression (Fig. 3c). In posterior lateral mesoderm explants, the combination of exogenous applied BMP2 and FGFs is required to induce full cardiac differentiation. Therefore, the inability of only
BMP2 to induce full cardiac differentiation might be caused by its inability to induce FGF expression in these explants (Fig. 3d). Restriction of ActRIIB receptor expression to anterior mesoderm (Lührman and Yutzey, 1999) might relate to the inability of posterior mesoderm to express FGF in response to BMP2.

The combination of BMP and FGF was thought to be sufficient to redirect the fate of non-precardiac mesoderm into the myocardial cell lineage (Lough et al., 1996). However, mesoderm from the most posterior region of the embryo could not be induced to differentiate into the cardiogenic lineage by BMP and FGF (Fig. 3b2; G. Di Rocco and A. Lassar, unpublished as mentioned in Marvin et al., 2001). Since posterior mesoderm of HH15 might contain precardiac cells, whereas the most posterior lateral mesoderm is likely to be devoid of precardiac cells (Fig. 3b), these findings might suggest that the inducing signal of BMP and FGF is not instructive, i.e. capable of changing the fate of cells, but permissive, i.e. promoting differentiation of already committed precardiac myocytes.

In mice, functional inactivation of BMP2 (Zhang and Bradley, 1996) FGF2, (Ortega et al., 1998), FGF10 (Sekine et al., 1999), BMP5 (Kingsley et al., 1992), BMP6 (Solloway et al., 1998), and BMP7 (Dudley et al., 1995; Luo et al., 1995) or the BMP receptors BMPRIIB (ALK-6; Yi et al., 2000), ActRIIA (Matzuk et al., 1995) and ActRIIB (Oh and Li, 1997) does not prevent the formation of a heart tube suggesting that they are not required for initial cardiac differentiation. It should be noted, however, that redundant functions of BMPs and their receptors in several tissues, including heart, have been suggested in studies on double mutant mice (Solloway and Robertson, 1999; Kim et al., 2001; Song et al., 1999; Matzuk et al., 1995; Oh and Li, 1997; Yi et al., 2000). BMP4 (Winnier et al., 1995), FGF4 (Feldman et al., 1995), FGF8 (Sun et al., 1999), BMP receptors ALK2 (Gu et al., 1999), ALK3 (Mishina et al., 1995), and BMPRII (Beppu et al., 2000) deficient embryos fail to form mesoderm, precluding detecting their potential role in myocardial differentiation. Nevertheless, BMP2 and FGF8 are expressed in the heart forming regions (Crossley and Martin, 1995; Winnier et al., 1995), suggesting that BMPs and FGFs are also involved in cardiomyogenesis in the mouse.

Since myocardium formation at both the anterior and posterior pole of the tubular heart is a continuation of the myocardium formation of the initial linear heart tube, the same factors are expected to be involved. Indeed, expression pattern of BMP and FGF suggest roles in the myocardium formation at the anterior and posterior pole. In outflow tract myocardium of the mouse, BMP2, 4, 6 and 7 mRNA is expressed (Jones et al., 1991; Kim et al., 2001; Lyons et al., 1990). In addition, FGF10 is expressed by cardiac precursor cells in the pharyngeal mesoderm and is switched off as they approach the heart (Kelly et al., 2001). In chicken, BMP2 and 5 mRNA is expressed in the outflow tract myocardium (Yamagishi et al., 2001; Waldo et al., 2001), whereas FGF8 has been found to be expressed in ventrolateral pharynx in both the ectoderm and to a lesser extent in the endoderm surrounding the mesodermal cells presumably contributing to the outflow tract myocardium (Waldo et al., 2001). In the inflow tract myocardium of the mouse, BMP5, 6
and 7 mRNA is expressed (Sollowa and Robertson, 1999; Kim et al., 2001), whereas in chicken BMP2 and BMP4 mRNA is expressed (Waldo et al., 2001; chapter 5).

The role of FGF10 in the myocardium formation at the anterior pole is not clear yet, but a role in cardiac precursor cell movement is suggested. This suggestion is supported by in vitro experiments in which cardiomyocytes that develop in chicken aortic sac culture change their epithelial phenotype into a mesenchymal, migratory phenotype upon addition of FGF10 (our unpublished observations). FGF8 is suggested to suppress differentiation but to stimulate proliferation of myoccardial cells. Neural crest cells (see below) that migrate into the pharynx were shown to block this signal (Farrell et al., 2001), allowing the differentiation of outflow tract myocardium.

**Wnt signaling pathways**

BMP2, BMP4 and several FGFs that are suggested to be involved in cardiac induction, are expressed in the primitive streak area of both chicken (Andree et al., 1998; Alsan and Schultheiss, 2002; Schultheiss et al., 1997; Karabagli et al., 2002; Shamim and Mason, 1999) and mouse embryos (Niswander and Martin, 1992; Winnier et al., 1995; Jones et al., 1991; Crossley and Martin, 1995). Although all mesodermal cells have been in contact with these BMPs and FGFs during gastrulation, only a subset in the anterior lateral region becomes myocardial. In addition, BMP and FGF are not able to induce myocardial differentiation in the most posterior lateral mesoderm and posterior primitive explants. Together these observations suggest that an additional factor is required for cardiac differentiation. This factor may play a role in actively inducing cardiomyogenesis in cardiac regions or preventing cardiomyogenesis in areas that do not become heart.

Studies of Tzahor and Lassar (2001) and Marvin and co-workers (2001) showed that Wnt signals are involved in suppressing cardiogenesis in non-cardiac mesoderm in chicken. Wnt’s are secreted signaling proteins of the highly conserved Wnt family that regulate cell fate decisions in vertebrates and invertebrates (reviewed in Cadigan and Nusse, 1997). Based on assays carried out with mammalian cell lines and *Xenopus* embryos, the Wnt family can be divided in the Wnt1 class including Wnt1, -2, -3, -3a, and -8, which mediate the signal through the canonical Wnt pathway involving β-catenin, and the Wnt5a class including Wnt4, -5a, -5b, -7b and -11, which mediate their signal through the non-canonical Wnt pathway involving protein kinase C (PKC) and Jun N-terminal kinase (JNK; Yamamoto et al., 1999; Wong et al., 1994; Du et al., 1995; Shimizu et al., 1997). In chicken, the Wnt1 class members Wnt3a and Wnt8c are expressed in the primitive streak and posterior lateral mesoderm at gastrulation stage (Fig. 6c and e; Marvin et al., 2001). Ectopic expression of Wnt3a or Wnt8c in precardiac mesoderm inhibited Nkx2.5 expression in vitro, and the beating of the precardiac mesodermal explants and the expression of Nkx2.5, GATA-4 and MHCs in vitro (Marvin et al., 2001). Crescent and dickkopf-1 (Dkk-1), which are specific inhibitors of the Wnt1 class/β-
catenin signaling pathway (Schneider and Mercola, 2001; Pera and Robertis, 2000; Glinka et al., 1998), were able to stimulate cardiogenesis in explanted (most) posterior lateral mesoderm as assessed by the presence of beating and MHC expression (Fig. 3d; Marvin et al., 2001). These findings indicate that Wnt signals belonging to the Wnt1 class (Wnt/β-catenin signaling) prevent cardiomyogenesis in the posterior mesoderm. Since crescent is expressed in anterior endoderm (Fig. 6d), crescent might be involved in suppressing the Wnt1 class signaling in the anterior mesoderm and thereby promoting cardiomyogenesis.

At HH9-10, the Wnt1 class members Wnt1 and Wnt3a are expressed in the open neural plate and dorsal neural tube (Tzahor and Lassar, 2001). The neural tube was able to inhibit the cardiogenesis in anterior paraxial mesendoderm and ectoderm (APMEE) explants from HH 8-9 chicken embryos that under standard conditions differentiate into cardiomyocytes. Supplementing Wnt1 or Wnt3a-expressing cells to APMEE explants could mimic the inhibitory effects of the neural tube. In addition, ectopic expression of Wnt-1 in one side of the heart-forming region of HH7 chicken embryos blocked expression of Nks-2.5. These findings suggest that Wnt1 class members Wnt1 and Wnt3a secreted from the neural tube prevent ectopic cardiogenesis.

Cardiogenesis was also inhibited in APMEE explants supplemented with noggin, which is expressed in the notochord (Tzahor and Lassar, 2001). Addition of Wnt/β-catenin antagonist Frzb-IgG and BMP2 was necessary to overcome the inhibitory effect.

Figure 7. Schematic drawing of a section through HH19 chicken embryo (Adapted from Tzahor and Lassar, 2001). Anterior paraxial mesendoderm with overlying ectoderm (APMEE) is outlined. Wnt1 and Wnt3a from the dorsal neural tube (NT) and noggin from the notochord (NC) inhibit heart formation in the dorsomedial anterior paraxial mesoderm.
of the neural tube and notochord on cardiogenesis in the APMEEE explants. Administration of BMP4 plus Fz2b-IgG to anterior paraxial mesoderm in vivo induced mesodermal cells to contribute to the heart and express vMHC (Tzahor and Lassar, 2001). These findings indicated that Wnt signals of the Wnt1 class from the dorsal neural tube together with BMP-antagonists from the notochord, noggin (and chordin) block ectopic cardiogenesis in anterior paraxial mesoderm (Fig. 7; Tzahor and Lassar, 2001).

Wnt signals were not only found to prevent cardiogenesis outside the heart forming fields, but also to stimulate cardiogenesis. Wnt11, which is a Wnt5A class member, is expressed at HH4 and HH5 in mesoderm overlapping the heart-forming fields (Fig. 6f; Eisenberg et al., 1997). Wnt11-conditioned medium was found to promote cardiogenesis in cultured posterior lateral mesoderm of HH5-6 chicken embryos (Eisenberg and Eisenberg, 1999). Marine Wnt11 conditioned medium induced GATA4, Nkx2.5, αMHC and ANP mRNA expression in P19 cells, indicating that the function of Wnt-11 in heart development has been conserved in higher vertebrates (Pandur et al., 2002). In addition, Wnt11 expression was observed in precardiac mesoderm of mouse embryos as well (Kispert et al., 1996). A recent study in Xenopus showed that Wnt11 signaling is independent of β-catenin and acts via activation of JNK, which appeared necessary for cardiogenesis (Pandur et al., 2002). Moreover, it was shown that activation of cardiogenesis by crescent and Dkk-1 is not only reached by inhibiting β-catenin signaling (Schneider and Mercola, 2001), but also by activating JNK signaling. (Fig. 6;
General introduction

Pandur et al., 2002). Taken together, these data indicate that induction of cardiomyogenesis requires low Wnt/β-catenin signaling activity and high Wnt/JNK signaling activity.

This JNK intracellular signaling cascade seems to be the integrator between Wnt and BMP signaling (Fig. 8), because besides Smad-mediated BMP signaling also JNK-mediated BMP signaling has been reported to be involved in cardiomyocyte differentiation (reviewed in Monzen et al., 2002).

As mentioned above crescent can induce ectopic expression of cardiac markers in most posterior lateral mesoderm in vitro (Fig. 3d), however, it cannot do so in vivo. This might indicate the presence of an additional repressor of heart formation that decays rapidly in explanted posterior lateral mesoderm or that is present in the removed endodermal and ectodermal cell layers. Similarly, both crescent and Dkk-1 cannot induce cardiogenesis in explanted posterior primitive streak tissue (Fig. 3d). Anterior endoderm can induce cardiogenesis in explanted posterior primitive streak tissue (Schultheiss et al., 1995; Marvin et al., 2001). This indicates that in addition to crescent, another factor that is expressed by the anterior endoderm is required to induce cardiogenesis in explanted posterior primitive streak tissue. This might be another Wnt/β-catenin antagonist, since posterior primitive streak tissue contains much higher levels of Wnt-3a and Wnt-8c than the posterior lateral mesoderm. Alternatively, it might be a member of the BMP or FGF family. However, BMP2, BMP4, FGF4 and FGF8 are already highly expressed in posterior primitive streak (Schultheiss et al., 1997; Karabagli et al., 2002). Moreover, addition of beads carrying BMP2 and FGF8, and cells expressing crescent do not induce expression of Nkx2.5 in posterior regions of the embryo in vivo (Alsan, Marvin and Schultheiss, unpublished result in Alsan and Schultheiss, 2002), indicating that in addition to the BMP, FGF and Wnt-signaling pathways an, as yet, unidentified signaling pathway/factor might be required for cardiogenesis (Fig. 6g).

The role of Wnt signaling in the myocardium formation at the anterior or posterior pole is not clear yet. However, presuming its location medial to the primary heart fields, the anterior heart field might be more exposed to the influence of cardiogenesis-inhibiting Wnt signaling from the neural tube than the posterior heart field (Tzahor andlassar, 2001), explaining its slightly later onset of cardiomyogenesis. By the folding of the embryo and the formation of the linear heart tube, the foregut becomes located in-between the anterior heart field and the neural tube. The anterior heart field might, therefore, no longer be under the influence of Wnt signaling from the neural tube, allowing cardiomyogenesis to occur.
Chapter 1

Cardiac cushion formation

Endocardium-derived mesenchyme

The formed tubular heart consists of two concentric layers of cells; the outer myocardial layer separated by cardiac jelly from the inner endocardial layer (De Jong et al., 1997). In the outflow tract and atrioventricular canal, JB3-expressing endocardial cells become hypertrophic, detach from their epithelial context, undergo an endocardial-to-mesenchymal transformation (EMT), and migrate into the cardiac jelly (Bolender and Markwald, 1979; Markwald et al., 1975, 1977; Wünsch et al., 1994). Myocardium of the atrioventricular canal and outflow tract induces this transformation of endocardium into mesenchyme by secretions of multicomponent complexes, called adherons (Fig. 9; Mjaatvedt and Markwald, 1989). Adherons comprise the ES (EDTA soluble) proteins fibronectin, transferrin, ES130, hLAMP-1 (heart lectin-associated myocardial proteins),...
hepatocyte growth factor and some other lower molecular weight proteins (Sinning et al., 1995; Isokawa et al., 1994; Krug et al., 1995; Mjaatvedt et al., 1999). Upon activation of the endocardium by myocardium, the endocardium and cushion mesenchyme become the signaling cells (Ramsdell and Markwald, 1997). In chicken, TGFβ2 and 3, and TGFβ type II receptor (TGFβRII) and TGFβRIII are found to have important roles in epithelial-mesenchymal transformation in the endocardial cushion (Ramsdell and Markwald, 1997; Potts et al., 1991, 1992; Barnett et al., 1994; Boyer et al., 1999; Brown et al., 1996, 1999; Nakajima et al., 1998). TGFβ2 and the TGFβRIII appeared to be critical for endothelial cell separation. TGFβ3 and the TGFβRII appeared to be critical to mesenchymal cell formation (Boyer and Runyan, 2001). The transcription factor Slug, which is expressed in the AV canal and is required for initial steps of EMT (Romano and Runyan, 1999), was shown to be an essential target for TGFβ2 signaling, but not for TGFβ3 signaling (Romano and Runyan, 2000). In mouse, only TGFβ2 is necessary for mesenchymal cell formation (Camenisch et al., 2002). Also BMPs are implicated in cushion formation. In chicken, BMP2 has been shown to act synergistically with TGFβ3 (Yamagishi et al., 1999). In mouse, BMP6 and 7 are suggested to play a role in epithelial-mesenchymal transformation, since BMP6/BMP7 double mutants display a pronounced delay in the morphogenesis of the outflow tract cushions (Kim et al., 2001). In chicken outflow tract, misexpression of noggin, which binds to BMP2 and 4 preventing receptor occupancy (Zimmerman et al., 1996), caused a decrease in the number of proliferating mesenchymal cells within the proximal cushions underscoring a role of BMP2 and 4 in cushion development (Allen et al., 2001). Other BMPs are also expressed in outflow tract or atrioventricular myocardium prior to cushion formation, including BMP4 and 5 (Jones et al., 1991; Solloway and Robertson, 1999), indicating a potential role in cushion formation. Conditional KO mice, in which only the cardiomyocytes from E10.5 onwards lack functional ALK3 (BMPRIA) display disturbed development of the trabeculae, compact myocardium, and ventricular septum, but also of the endocardial cushions indicating ALK3-dependent cross-talk of the cardiomyocytes to the mesenchymal cells of the cushions (Gaussin et al., 2002).

After the initial epithelial to mesenchymal transition, the endocardial-derived mesenchymal cells continue to proliferate, stimulated by growth factors such as FGF2 (Choy et al., 1996). The mesenchyme that has reached the sub-myocardial cushion region, reduce proliferation and begin to selectively express genes associated with differentiation (Mjaatvedt et al., 1999).

In transcription factor Sox4 (Ya et al., 1998) and NF-ATc (De la Pompa et al., 1998) deficient mice the endocardium-derived mesenchyme appears to be affected. SOX4 is expressed in endocardium-derived mesenchyme and Sox4-deficient mice suffer from aberrant transformation of the endocardial cushions into semilunar valves and from lack of fusion, usually resulting in common trunk. However, the development of the
atrioventricular cushions is not visibly affected, suggesting that the development of the cushions of the outflow tract and atrioventricular canal is differentially regulated. This is supported by the observations that outflow tract and atrioventricular cushions are differentially affected in mice deficient for BMP6/BMP7 (Kim et al., 2001) and tolloid-like 1 gene (Clark et al., 1999). NF-ATc is specifically expressed in endocardial cells. In NF-ATc deficient mice the cardiac valves are affected in both outflow tract and atrioventricular canal, suggesting that some overlap exists in the regulation of the cushion formation in the outflow tract and atrioventricular canal (De la Pompa et al., 1998).

**Neural crest-derived mesenchyme**

Cardiac neural crest cells migrate from the cranial neural folds between the midotic placode and the caudal limit of somite 3. These cells contribute mesenchyme to the aorticopulmonary septum and form two prongs of condensed mesenchyme that extend to the future level of the semilunar valves. In the proximal outflow tract, these neural crest-derived cells become dispersed in the endocardial ridges and flanking myocardium (Poelmann et al., 1998; Waldo et al., 1998; Ya et al., 1998; Creazzo et al., 1998). Cardiac neural crest ablation in chick embryos causes mostly persistent truncus arteriosus (PTA), but also double outlet right ventricle (DORV) and ventricular septal defects (VSD; Kirby et al., 1985), indicating an important role for neural crest in outflow tract septation. Studies suggest that cardiac neural crest cells in mouse and chick have similar migration patterns and serve the same function (Waldo et al., 1999; Jiang et al., 2000). Remarkably, numerous distinct transgenic mice show a septation defect in the outflow tract associated with absence or improper functioning of the cardiac neural crest, being: (1) the Splotch mice, in which the transcription factor Pax3 is deficient (Conway et al., 2000), (2) the Patch mice, in which a chromosomal deletion resulted in the lose of the αPDGFαR gene and in the alteration of the domain of expression of c-kit gene (Schatteman et al., 1995; Duttlinger et al., 1995; Wehrle-Haller et al., 1996), (3) the PDGFα receptor deficient mice, which in contrast to the patch mouse only the PDGFα receptor gene is deleted (Soriano, 1997), (4) RXR alpha (Gruber et al., 1996), (5) neurofibromin-1 (Nf-1; Brannan et al., 1994), (6) neurotrophin-3 (NT-3: Donovan et al., 1996), (7) endothelin-1 (ET-1; Kurihara et al., 1995), and (8) semaphorin 3C deficient mice (Feiner et al., 2001). In the outflow tract of the trisomic 16 (Ts16) mouse, the organization of the neural crest population was severely perturbated (Waller III et al., 2000). Smooth muscle actin-positive mesenchymal cells, which presumably are derived from the neural crest do not penetrate into proximal outflow tract cushions, but align more distally with the outflow tract myocardium inducing ectopic myocardium formation (Waller III et al., 2000).

A recent study suggests that the arrival of cardiac neural crest cells in the caudal pharyngeal arches is also required for normal differentiation at the anterior pole (Yelbuz
et al., 2002). After neural crest-ablation, chicken embryos displayed a shorter outflow tract caused by a reduction in the number of myocardial cells added to the anterior pole, which resulted in abnormal looping (Yelbuz et al., 2002).

The contribution of the neural crest to the mature heart is limited. In the mouse, they occupy the fibrous region between the valves and form smooth muscle cells in the proximal coronary arteries (Jiang et al., 2000).

**Epicardium-derived mesenchyme**

Another population of mesenchymal cells that populates the atrioventricular cushions is derived from the epicardium (Gittenberger-de Groot et al., 1998; Männer, 1999). The epicardium develops from the proepicardium, which is located caudally to the heart (Männer et al., 2001). The proepicardium forms a cauliflower-like mesothelial structure protruding from the coelomic wall into the pericardial cavity. Upon attachment to the heart, the proepicardial cells start to envelop the heart, forming the epicardium. In between the epicardium and myocardium, mesenchymal cells are deposited that will, in addition to the atrioventricular cushion mesenchyme, contribute to the cardiac fibroblasts, and the endothelial and smooth muscle cells of the coronary arteries (Fig. 10; Mikawa and Gourdie, 1996; Dettman et al., 1998; Pérez-Pomares et al., 1997, 1998, 2002;

![Epicardium](image)

**Figure 10.** Schematic diagram depicting the fate of the epicardium-derived mesenchyme (Adapted from Morahito et al., 2001). Subepicardial mesenchyme (MesC) is formed by epicardial-mesenchymal transformation. This mesenchyme gives rise to coronary vascular smooth muscle cells (CVSMC), coronary endothelial cells (En), perivascular fibroblasts (PerIF), or intermyocardial fibroblast (MyoF).
Gittenberger-de Groot et al., 1998; Männer et al., 1999; Vrancken Peeters et al., 1999); VCAM-1 (Kwee et al., 1995), α4-integrin (Yang et al., 1995) and Wilms' tumor-1 (Moore et al., 1999) deficient mice show a lack of proper epicardial formation and have an absence of epicardial-derived mesenchymal cells. In POG-2 deficient mice an intact epicardial layer is formed but no epicardial-derived cells are formed (Tevosian et al., 2000). These mice lack coronary vasculature and die at midgestation with a cardiac defect characterized by a thin ventricular myocardium.

Similarly, epicardial outgrowth inhibition in avian embryos resulted in abnormalities of the compact myocardial layer and coronary vasculature (Gittenberger-de Groot et al., 2000). In addition, septation of the embryonic heart was disturbed.

Mediastinum-derived mesenchyme

The mesenchyme of the dorsal mesocardium and its protrusion into the lumen of the heart, i.e. spina vestibuli, are considered to originate from an extra-cardiac source. Controversy exists, however, about the origin of the mesenchymal cap of the primary atrial septum. Endothelial-to-mesenchymal transformation has been found in the mesenchymal cap (Arrechedera et al., 1987; Gerety and Watanabe, 1997), indicating endocardial contribution to the mesenchyme. In addition, a substantial cap of mesenchyme was still present on the leading edge of the primary atrial septum in Ts16 mice that lacked their spina vestibuli (Webb et al., 1999). These observations suggest that the mesenchyme of the cap of the primary atrial septum is derived from the endocardium. On sections of human heart, however, the mesenchyme of the spina vestibuli and the cap of the primary atrial septum can be distinguished from the mesenchyme of the endocardial cushions on the basis of specific antibody staining (Wessels et al., 1996). Based on this difference in gene expression an extra-cardiac origin of the mesenchymal cap of the primary atrial septum has been suggested.

Myocardium formation in intra- and extra-cardiac mesenchyme

The heart tube is formed by a continuous addition of myocardium to the anterior and posterior pole of the heart. At the venous pole this myocardial addition continues in the extra-cardiac mesenchyme forming the caval and pulmonary myocardium. In order to establish a four-chambered heart, supporting two separate blood circulations, the tubular heart remodels and becomes septated by fusion of the cardiac cushions, the mesenchymal cap of the primary atrial septum, the spina vestibuli and the ventricular septum (Wessels et al., 1996, 2000; Webb et al., 1998; reviewed in Lamers and Moorman, 2002). This results in the formation of mesenchymal septa. However, in the adult heart, the part of the outlet septum that is incorporated into the ventricular component is muscular (Fig.
11). Thus, the mesenchyme of the proximal outlet septum has to be replaced by cardiomyocytes. The importance of myocardium formation during septation is suggested by studies in which perturbed myocardium formation was correlated with congenital outflow tract defects (Sanford et al., 1997; Waller et al., 2000; Bartram et al., 2001). Myocardium formation in intra- and extra-cardiac mesenchyme has been referred to in previous studies (Okamoto et al., 1981; De la Cruz et al., 1989; Franco et al., 1999; Lamers et al., 1995; Ya et al., 1998). However, only recently this process has received more specific attention (Van den Hoff et al., 1999; Moorman et al., 2000; Mjaatvedt et al., 1999). Myocardium formation in the outlet septum of the chicken appeared to be a relative late process in cardiac septation, being initiated around HH28 and being completed around HH38 (Van den Hoff et al., 1999). Using an *in vitro* explant assay, it was found that the outflow tract was "myocardialization-competent" at all stages tested (HH16-HH30), but formed spontaneously extensive myocardial networks from HH20 onward. Myocardial network formation was induced by factors produced by, most likely, the non-myocardial component of the outflow tract. Ventricular myocardium, however, was not found to form myocardial networks *in vitro* and to produce factors capable of inducing myocardial networks. In this study, the myocardium formation was suggested to take place by growth of existing myocardium into the mesenchymal outlet septum (myocardialization).

**Figure 11.** The initial mesenchymal outlet septum becomes muscularized during subsequent development. The outlet septum forms by fusion of the mesenchymal cushion tissue. In the adult heart, the outlet septum is muscular indicating that the mesenchymal cells are replaced by cardiomyocytes.
Aim of this thesis

The aim of this thesis is to obtain more insight into the process of myocardium formation after the initial formation of the linear heart tube and into the mechanisms underlying this process. In chapter 2, the study of myocardium formation in the outflow tract of the chicken heart (Van den Hoff et al., 1999) was completed by describing myocardium formation in the atrioventricular canal and venous pole. In chapter 3, the development of the \textit{in vitro} assay for mouse explants is described. In chapter 4, the myocardium formation in the intra- and extra-cardiac mesenchyme in the mouse has been studied as a basis for studies in transgenic mouse models. In chapter 5, the αSMA expression in the myocardium forming regions of the heart is described. In chapter 6, the potential of the proepicardial cells to differentiate into myocardial cells is investigated. In chapter 7, preliminary data providing more insight in the mechanisms underlying myocardium formation is presented. At the end, a short summary is given.

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General introduction

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