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Alk2 BMP receptor domains in developing chick heart : immunohistochemical mapping

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ALK2 BMP RECEPTOR DOMAINS IN DEVELOPING CHICK HEART: IMMUNOHISTOCHEMICAL MAPPING

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ABSTRACT

ALK2 BMP RECEPTOR DOMAINS IN DEVELOPING CHICK HEART:

IMMUNOHISTOCHEMICAL MAPPING

By Ricardo Leitão

The heart is one of the first organs to develop during the embryogenesis. While heart development is not the result of a single molecular pathway, the bone morphogenetic protein (BMP) pathway has earned relevance over its influence on heart formation. Recent studies demonstrate that BMPs and their receptors are likely to be extremely important players involved in the creation and differentiation of the primary heart-forming regions, heart tube formation, elongation, looping, septation and valvulogenises. In general, myocardial BMPs bind to endocardial BMP receptors to activate a signaling cascade that culminates in the production of cardiac-related proteins. Of the different BMP receptors known to date, Alk2 is one of the most influential in heart development. Alk2 transcripts have been mapped to regions where anatomical heart structures arise, and their ablation has consequences remarkably more severe than those of ablation of other tested BMP receptors. However, despite their importance, no expression studies have yet been performed. In this study, we sought to determine the localization of Alk2 proteins via immunohistochemistry. Our results demonstrate that, in an embryo that is approximately 80 hr old, Alk2 proteins are

preferentially expressed around the outflow tract, the developing endocardial cushions, throughout the septum primum, and possibly scattered throughout the embryonic ventricle.

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The research underlying this thesis was the result of the culmination of joint efforts, in which, teachers, advisors, friends, and the scientific community outside this institution played an important role. Without them, this research would not have been possible and I would not have had the exposure to the broad information available.

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THIS THESIS IS DEDICATED TO ALL MY FAMILY
FOR THEIR SUPPORT AND ENCOURAGEMENT

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INTRODUCTION

The heart is one of the first organs to develop during the embryonic life of any complex living being. Its function, the pumping of blood throughout all body tissues, is simple and essential. To perform such a simple function, the heart develops a complex network of channels, tubes that loop and bend, valves that shunt, and cells that contract. Heart formation is complex and far from being completely understood.

The work herein was designed with a vision to understand the control of this complex development. Bone morphogenetic proteins (BMPs), named initially for their role in bone formation, have more recently been found to act as one of the major classes of molecules controlling heart formation. According to Wijk et al. (2007), BMPs are the inducers of cardiac differentiation for a number of steps in heart development. They are thought to participate in differentiation of the heart-forming regions, the recruitment of cardiomyocytes at the distal borders of the heart, and septovalvular development (Wijk et al., 2007). So far, however, studies have not defined the complete role and function of BMPs. According to Délot et al. (2003), the BMP2 and -4 molecules are known to be present exclusively in the region of atrioventricular cushion formation. Other studies report the presence of BMPs in valvular regions such as the outflow tract (OFT) and atrioventricular canal. Understanding the expression of BMP receptors is crucial to elucidate such controversial information. Expression maps, when

combined with the understanding of the anatomical heart development and the results of knockdown mutational studies, will allow us to understand the molecular functions of BMPs.

As the roles of various BMPs and their associated BMP receptors slowly emerged over the last decade, it has become increasingly important to establish the cardiac expression domains over which the various BMPs act and find how they change over the course of development. The structural similarity of BMPs, however, has rendered specific gene probing by in situ hybridization both complicated from a probe design standpoint and technically difficult to achieve. Furthermore, interpretation of the often conflicting data in the literature is not straightforward, due to the inability to estimate the amount of any particular BMP mRNA actually translated or released from a cell.

To circumvent some of these problems, we sought to use immunohistochemistry to define the Alk2 expression domain in the developing chick heart. This methodology, however, is fraught with its own technical difficulties; besides the localization of the receptor in heart development, this research was designed to optimize the conditions for this methodology.

ABBREVIATIONS

- aPBA - 1% acetylated BSA in 1xPBS
- 1X-DPBS - calcium/magnesium free Dulbeccos modified phosphate buffered saline
- AVS - atrioventricular septum
- Ab(s) - antibody(s)
- ASP - atrial septum primum
- AV - atrioventricular
- AVC - antrioventricular canal
- BCIP/NBT - 5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium
- BMP(s) - Bone morphogenetic protein(s)
- BSA - bovine serum Albumin
- CNC - cardiac neural crest
- CPM - cranial paraxial mesoderm
- diH2O - distilled water
- DPC - days post coitum
- EMT - epithelial to mesenchymal transformation
- FGF(s) - Fibroblast growth factor(s)
- HH - Hamburger-Hamilton
- HIER - heat induced epitope retrieval
- IHC - immunohistochemistry
- Mef2a - Muscle enhancer factor
- NC - neural crest
- OFT - outflow tract, conus arteriosus
- PBA - 1% BSA in 1xPBS
- PBS - Phosphate Buffer Saline
- PFA - 4% paraformaldehyde
- PHF - primary heart fields
- PIER - proteolytic induced epitope retrieval
- SAHF - secondary/anterior heart fields
- SMAD - mothers against decapentaplegic homolog
- TGF - Transforming growth factor
- Tob - Osteoblast α-proliferative protein

1 - LITERATURE REVIEW

1.1 - BMP Pathway

More than twenty bone morphogenetic proteins (BMPs) which belong to the transforming growth factor (TGF) ß superfamily of signal transducers are known. Similar to other TGFß signaling molecules, BMPs initiate an intracellular response via an interaction with at least two of the three types of TGFß serine/ threonine kinase receptors (Taimor et al., 2006; Chen et al., 2004). According to Taimor et al. (2006), a set of five type II receptors that exist in a constitutively active form have been identified. Such receptors interact with type I receptors upon ligand binding. Apparently, constitutively active type I and II receptors are not individually sufficient to transduce an effective signal (Miyazono et al., 2005). Most likely, conformational changes in type II receptors upon ligand binding predisposes their interaction with type I receptors. Type I receptors are then phosphorylated in a GS (Gly-Ser) domain (Miyazono et al., 2005). As a cooperative positive feedback function, type I receptors enhance the capacity of BMPs to bind type II receptors (Liu et al., 1995).

A total of seven type I receptors and five type II receptors exist. Three of the seven type I receptors and three of the five type II receptors are known to bind BMPs. BMPs exhibit higher affinity to type I molecules (Taimor et al., 2006; Wijk et al., 2007). Type I receptors are divided into two categories, type IA and IB (Wijk et al., 2007; Chen et al., 2004). BMP receptors of type IA include only

Alk2 (or ActR-IA, ACVRI), which is the focus of the present study. Type IB receptors are made of two different receptors, Alk3 (BMPR-IA, BRK-1) or Alk6 (BMPR-IB, BRK-2) (Chen et al., 2004; Wijk et al., 2007) (Fig. 1).

	Type I Receptors	Type II Receptors
IA	• ACVR1 or Alk2 or ActRI	• BMPR2 or BMPRII or BRK-3
IB	• BMPR1A or Alk3 or BRK-1	• ACVR2A or Cact-Riia
	• BMPR1B or Alk6 or BRK-2	• ACVR2B or ActRIIIB

Fig. 1. Type I and II BMP receptors. Type I receptor subtypes are indicated on the left (IA, IB). The multiple names for each entry (separated by "or") are synonyms found in the literature.

Each group of receptors is responsible for signal transduction via SMAD activation (a SMA and MAD (mothers against decapentaplegic) homolog). However, each group of receptors activates different SMAD isoforms because different ligands bind with different affinity to type I/II complexes. Upon interaction with their respective type II receptors (TBRII and ActR-IIB), SMAD2 and -3 are activated (Taimor et al., 2006).

SMAD activation occurs as the result of a complex balance between BMP ligand/receptor interaction and the presence or absence of blocking peptides. A range of proteins are known to have negative effects on the BMP response.

SMAD6 (also designated iSMAD) has notable effect in binding BMP receptors of the type I class and preventing phosphorylation of downstream R-SMADs (Chen et al., 2004). Other proteins such as osteoblast α-proliferative protein (Tob) and Smurf1 (a U3 ubiquitin ligase) can target and bind SMAD1 and -5 and act as SMAD inhibitors (Chen et al., 2004).

SMADs, the downstream effectors of TGFß superfamily ligands and their receptors are proteins composed of eight distinct protein subunits. SMAD proteins are commonly organized into three classes: the receptor-activated R-SMADs which include SMAD1, -2, -3, -5, and -8, the co-mediator Co-SMAD (SMAD4), and the SMAD inhibitors (also designated iSMAD) which include SMAD6 and -7 (Ishida et al., 2000; Taimor et al., 2006). According to Taimor et al. (2006), all SMADs and nearly all of their isoforms in the cardiovascular system have been successfully identified.

R-SMADs are mainly localized in the cytoplasm and are phosphorylated upon type I receptor activation. Thus, they are named receptor-regulated SMADs. As a result of this phosphorylation, R-SMADs dimerize and interact with common-partner SMADs (Co-SMADs) which are found in the cytoplasm and nucleus (Taimor et al., 2006; Miyazono et al., 2005). During this process, only three of the five R-SMADs are able to interact with BMP-activated receptors. The other two are activated by activin/TGFß members (Miyazono et al., 2005). BMP-responsive SMADs include SMAD1, -5 and -8 and are commonly designated as

BR-SMADs (Miyazono et al., 2005). SMAD2 and -3 preferably respond to TGFß signals.

Heterotrimer complexes of R and Co-SMADs translocate to the nucleus to activate gene transcription (Taimor et al., 2006). Gene regulation can occur via a multitude of mechanisms that include: *i)* direct binding of SMAD complexes to DNA at a GTCT-binding site to which SMADs bind with low affinity and, *ii)* recruitment and interaction with DNA-binding proteins (Ishida et al., 2000; Taimor et al., 2006). Possible DNA binding proteins including p300/CBP for histone acetylation or co-repressors and co-activators such as FAST1, c-Jun-c-Fos, or PEBP2/Runx (Ishida et al., 2000). The Gata4 transcription factor is crucial to direct SMAD-mediated activation of Nkx2.5 during BMP activation of SMADs. An enhancer element in the Nkx2.5 gene containing several SMAD1, -4 and Gata biding sites was recently discovered to confirm this requirement (Taimor et al., 2006). The extent of BMP pathway activation is then controlled by a feedback loop over SAMD6. Studies have shown that constitutively active BMP receptors activate the transcription of reporter genes associated with SMAD6 promoters (Takase et al., 1998; Ishida et al., 2000). Both cardiomyocyte formation and endocardial cushion formation demonstrate this type of feedback (Wijk et al., 2007). During cushion formation, feedback occurs during over-expression of the BMP receptor. In turn, Alk2 causes over-expression of SMAD6 and knockout of SMAD6 results in hyperplastic valves (Chen et al., 2004).

Fig. 2 is a representation of the BMP pathway.

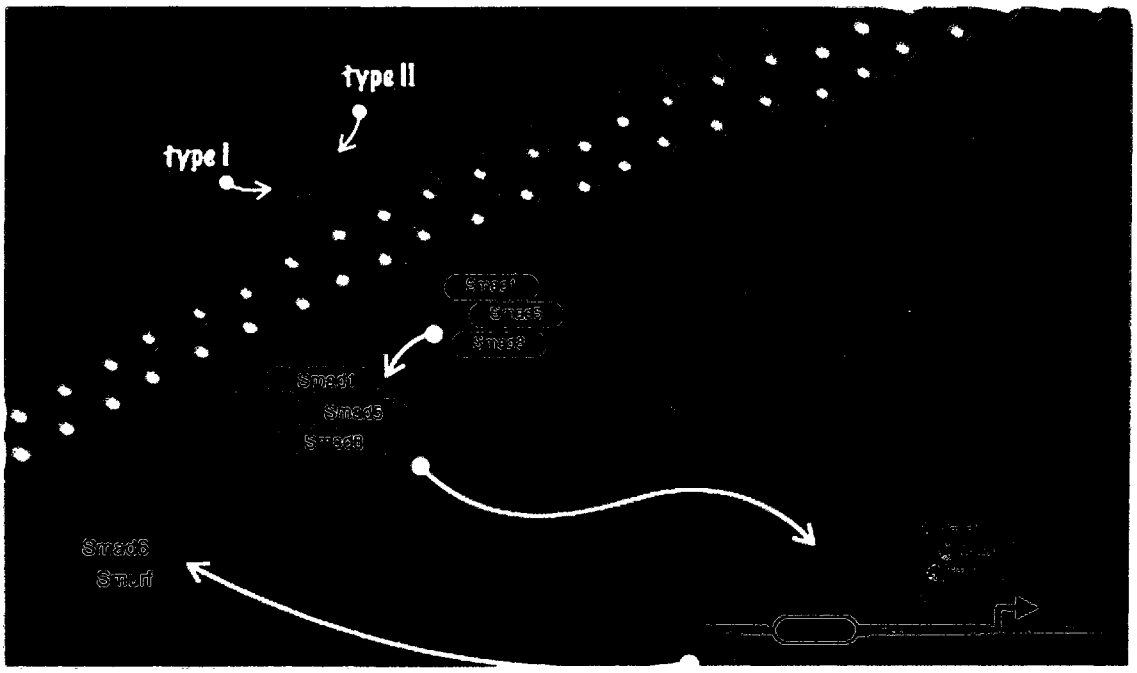

Fig. 2. BMP signaling pathway. Generic representation of a BMP pathway. Color legend: pink, BMP ligand; green, BMP receptors of type I and II; blue: intracellular proteins (Smads and Smurf1).

1.2 - Heart Formation and its Relationship with the BMP Pathway

1.2.1 - Genetic Mechanism Behind Heart Tube Formation

Chicken heart development starts at Hamburger-Hamilton (HH) stage 3 with the migration of cells through the primitive streak, the embryonic midline of the chick (Wijk et al., 2007; Ramsdell et al., 2005). The first few mesodermal

cells, which according to Tirosh et al. (2006) are splanchnic mesoderm, become cardiac progenitor cells (Wijk et al., 2007; Ramsdell et al., 2005). These cells accumulate to form the primary heart fields (PHF) (Ramsdell et al., 2005). The union of the lateral primary heart fields at the rostral end of the embryo results in the formation of the cardiac crescent, that arcs anteriorly around the midline of the embryo (Tirosh et al., 2006; Latif et al., 2006).

Current evidences suggest that mesodermal cells in the midline are prevented from differentiating into cardiac mesoderm due to Wnt signaling molecules. These molecules, such as Wnt1, -3a, and -8 are secreted by the neural tube (Wijk et al., 2007; Tirosh et al., 2006; Gilbert, 2003). In contrast, cardiac mesoderm generation is stimulated in the anterior lateral domains by BMPs (especially BMP2) and fibroblast growth factors (FGFs), especially FGF8. Such BMPs are secreted from the underlying anterior endoderm (Gilbert, 2003; Alsan and Schultheiss, 2002). Wnt inhibitors such as Dickkopf, Cerberus, and Crescent, may also be involved (Gilbert, 2003). Numerous studies suggest that Wnt signaling must be repressed in the two primary heart fields and the cardiac crescent for normal cardiogenesis to occur (Marvin et al., 2001; Tzahor, 2007; Tzahor and Lassar, 2001; Gilbert, 2003).

In situ hybridization studies indicate that many BMPs (BMP2, -4, -5, and -7) are expressed at varying degrees in the lateral plates and in the general region of the cardiac crescent (Somi et al., 2004). However, studies have shown

that the BMPs acting on the mesodermal cardiac precursors of the primary heart fields do not come from the mesoderm itself. BMPs are released from the underlying endoderm and overlying ectoderm (Wijk et al., 2007; Somi et al., 2004). BMP4 and -7 appear to be generated from the ectoderm and BMP2 and 5 from endoderm. The idea, however, that the heart field mesoderm responds only to BMPs and does not generate them itself remains in question. One study indicated the presence and possible production of BMP2 in the mesoderm (Andrée et al., 1998). Also, later in development, the mesoderm of the definitive heart tube is able to synthesize and secrete BMPs (Somi et al., 2004; Andrée et al., 1998; Schultheiss et al., 1997). Fig. 3 depicts the localization of BMP2, -4, -5, and -7 transcripts at different stages of chicken embryonic development, as demonstrated by Somi et al. (2004).

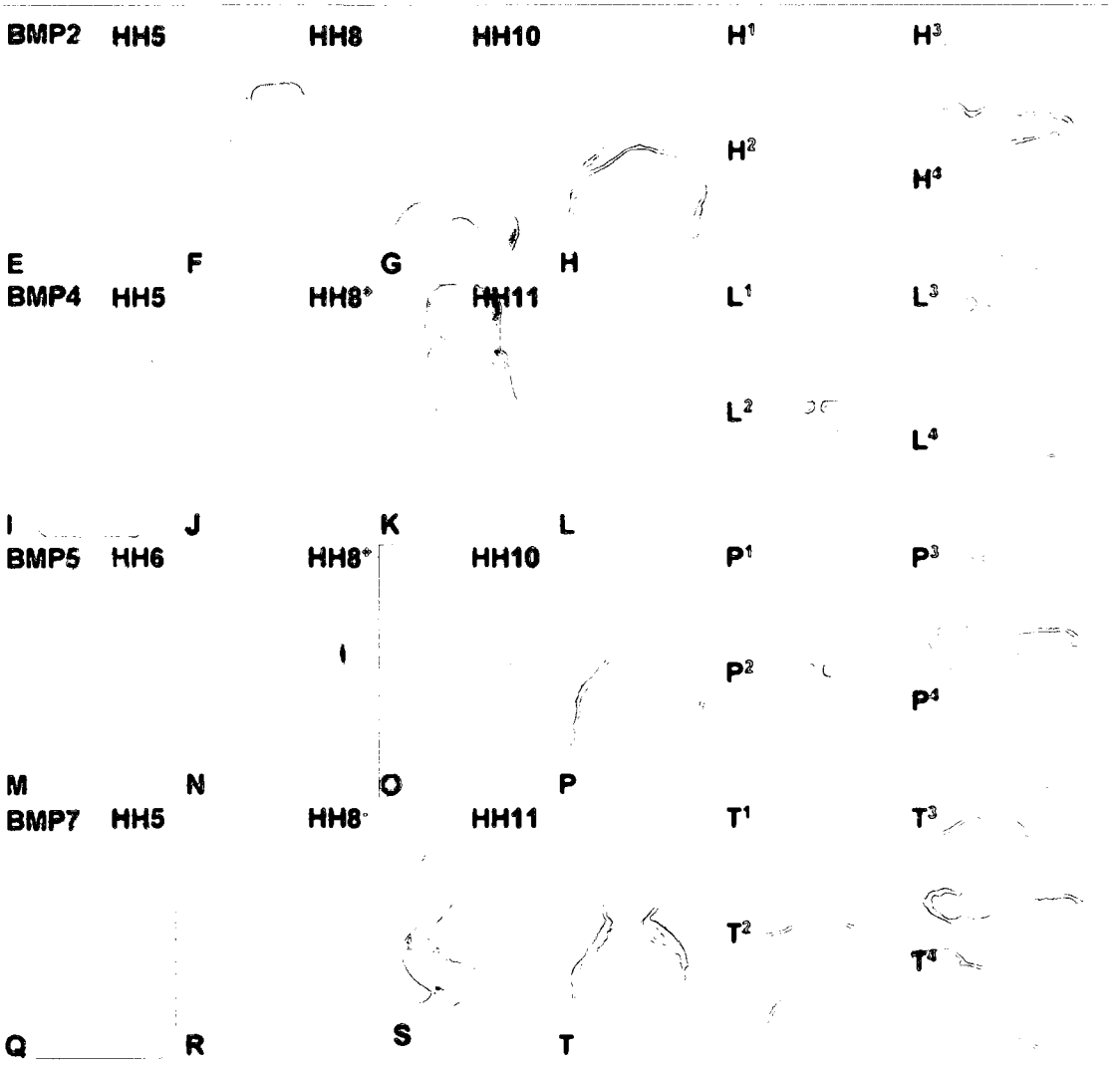

Fig. 3. In situ hybridization of BMP2, -4, -5, and -7 transcripts during formation of the cardiac crescent and heart tube. Transcripts are seen to co-localize in anterolateral regions coincident with the future heart-forming zones (detected via immunohistochemistry using anti α-troponin antibodies. Later in development, BMPs are seen throughout the chick heart tube. The right half of this figure depicts vertical and transversal sections of the stage 10 embryo. Reprinted from Somi et al. (2004) with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

As shown in Fig. 3, BMPs are expressed throughout development of the cardiac crescent and later on the heart tube. The binding of the different BMPs to their type I and II receptors initiates signal transduction cascades. This results in gene expression changes. As early as HH stage 4 or 5 of development, for example, BMPs drive the activation of the genes encoding Nkx2.5 and Gata4 and -5 transcription factors (Gilbert, 2003; Schlange et al., 2000; Schultheiss et al., 1997; Wijk et. al. 2007; Laverriere et al., 1994). By stages 5 to 7, the activation of a large number of transcription factors occurs, including Mef2, SRF, Tbx2, Tbx3, Tbx5, Tbx20, and dHAND (Wijk et al., 2007; Yamada et al., 2000; Buchberger et al., 1999; Srivastava et al., 1995). These molecules not only co-localize with those of BMPs (Fig. 4-8) but their expression can be induced with BMPs in explants of tissue that would otherwise not produce such molecules (Schlange et al., 2000; Schultheiss et al., 1997).

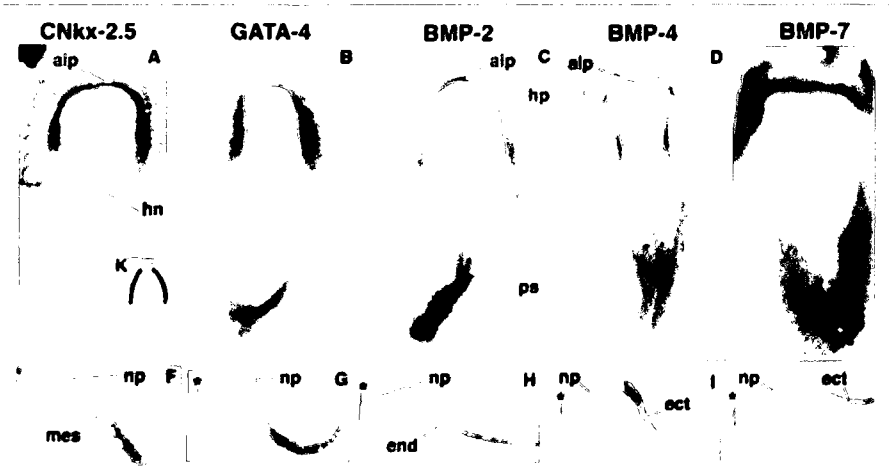

Fig. 4. Co-localization of BMPs and downstream genes. Demonstration of co-localization of BMP2, -4, and -7 with Nkx2.5 and Gata4 via whole mount in situ hybridization in stage 6 chick embryos. Aip, anterior intestinal portal; ect, ectoderm; end, endoderm; hn, Hensen's node; mes, mesoderm; np, neural plate: *, neural groove. Reprinted from Schultheiss el al. (1997), with permission from Cold Spring Harbor Laboratory Press.

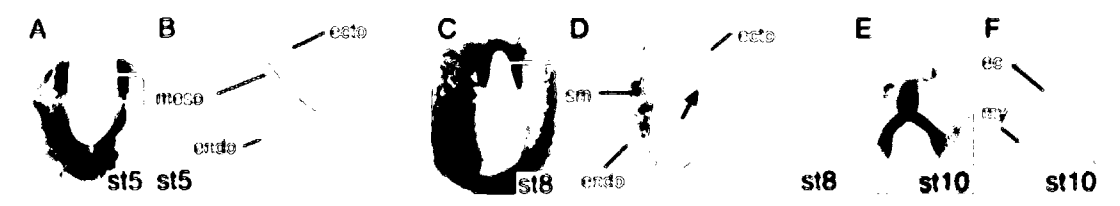

Fig. 5. *In situ* hybridization using a Tbx20 probe. Lateral plate mesoderm (A + B) contains Tbx20 transcripts. Such transcripts are present in future stages including in the formed heart tube (E+F). ec, endocardium; ecto, ectoderm; endo, endoderm; meso, mesoderm; my, myocardium; sm, smooth muscle. Reprinted from Yamagishi et al., 2004 with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

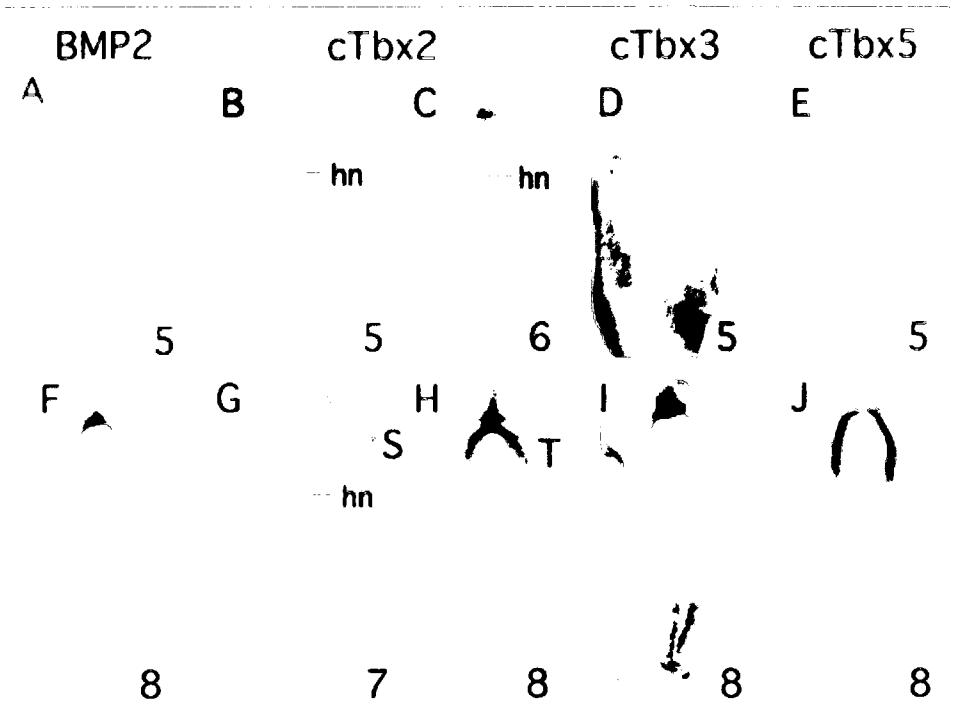

Fig. 6. *In situ* hybridization of BMPs and Tbx genes. Chick development HH stage 5 to 8. Ventral side up. "A) The expression of BMP2 was observed prior to that of cTbx2 in anterior lateral region of the embryo at stage 5. (B) cTbx2 expression was first detected at stage 5, in the crescent shaped anterior lateral cardiogenic region. (C) At stages 6, cTbx2 transcript levels were enriched in most of the anterior portion of the cardiogenic region. (D) cTbx3 was also in the cardiac crescent region at stage 5 and in the most posterior region of the embryo. (E) cTbx5 expression was restricted to the cardiogenic region at stage 5. (G) cTbx2 expression was enhanced in the nascent bilateral cardiac tubes by stage 8 (H). (F) BMP2 was expressed in cardiac tubes at stage 8 and (I) cTbx3 as well. All BMP2, cTbx2, and cTbx3 were expressed in a posterior part of primitive streak; in contrast, (J) cTbx5 expression was highly restricted in bilateral cardiac tubes" (Yamada et al., 2000). Hn, Hensen's node. Reprinted from Yamada et al. (2000), with permission from Elsevier.

With respect to Mef2a (Muscle enhancer factor), symmetrical expression in the nascent heart tube (pre-cardiac mesoderm) was seen by stage 8. Full expression in the formed tube was seen at stage 11 (Fig. 7) (Buchberger et al., 1999).

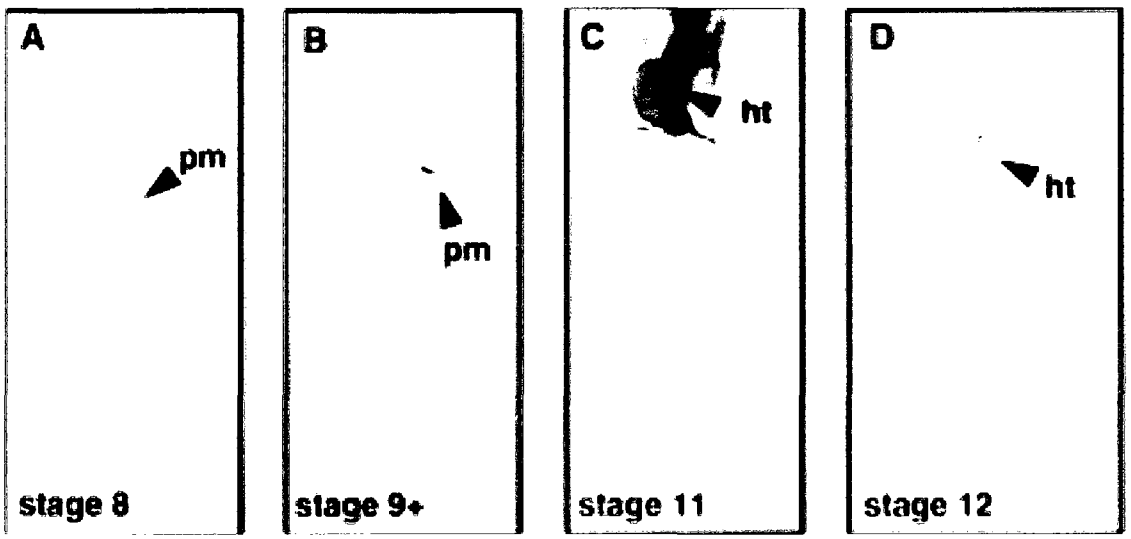

Fig. 7. *In situ* hybridization of Mef2A. Pre cardiac mesoderm and heart tube showing Mef2A transcripts. ht, heart; pm, precardiac mesoderm. Reprinted from Buchberger et al. (1999), with permission from Springer Science and Business Media.

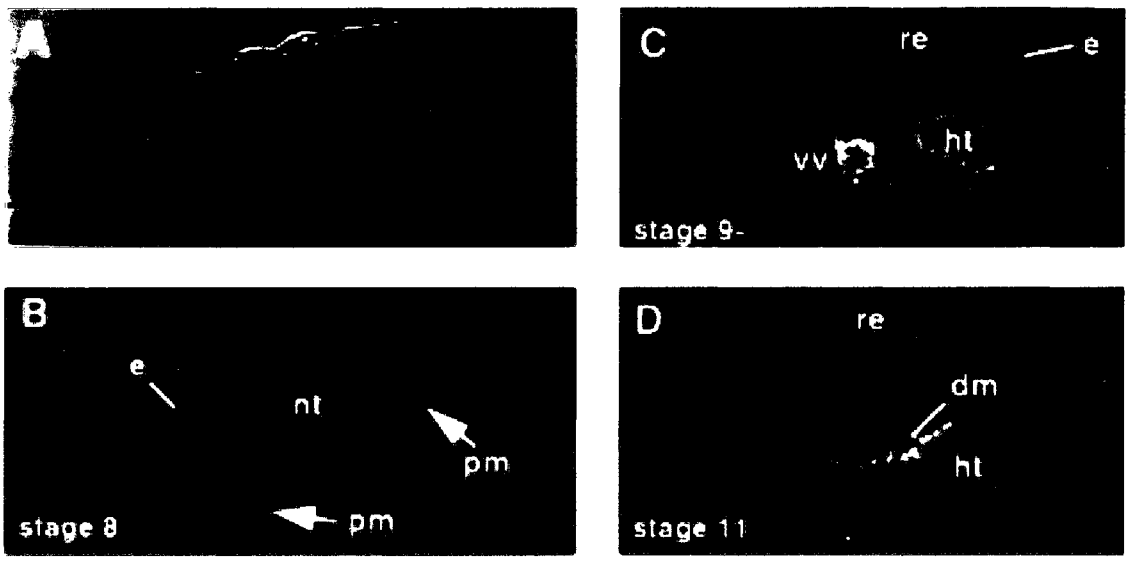

Fig. 8. *In situ* hybridization of Mef2A. 10μm section of the embryos show above by phase contrast (Panel A) and dark field (Panels B-D) imagery. Mef2A is seen slightly at the pre-cardiac mesoderm (pm) and later strongly at the heart tube (ht). dm, dorsal mesocardium; nt, neural tube; re, rhombencephalon; vv, vitelline vein. Reprinted from Buchberger et al., (1999), with permission from Springer Science and Business Media.

As shown in Fig. 9, dHAND and eHAND (members of the bHLH family of transcription factors) are also expressed in the developing heart (Srivastava et al., 1995). Like Mef2A, these two transcription factors may also contribute to the muscular development of the heart.

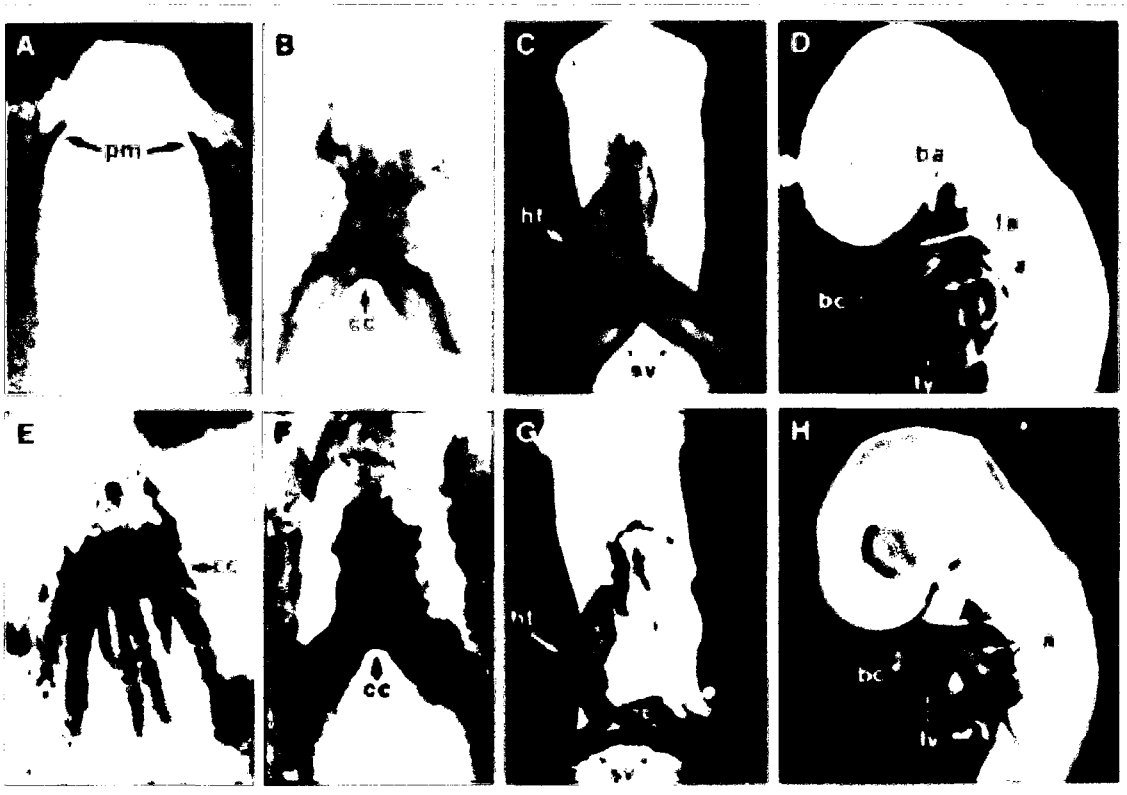

Fig. 9. *In situ* hybridization of dHAND and eHAND. *In situ* hybridization of dHAND (top panels) and eHAND (bottom) in developing mice embryos. A: stage 8-; E: stage 8+; B+F - stage 9; C+G - stage 10; D+H - stage 15. a, atria; ba, brachial arch; bc, bulbo cordis; cc, cardiac crescent; ht, heart tube; lv, future left ventricle; pm, precardiogenic mesoderm; sv, sinus venosus; ta, truncos arteriosus. Reprinted from Srivastava et al., (1995), with permission from the American Association for the Advancement of Science.

The activation of all these transcription factors will ultimately culminate in the expression of proteins that are specific for cardiac muscle, such that by HH stage 10 (33-38 hours of development) the heart starts to beat (Wijk et al., 2007;

Gilbert, 2003; Hill, 2008). Elongation and thickening of the heart tube then probably occurs by recruitment of more cardiomyocytes, at least in part due to the presence of BMP2 and/or BMP7 and FGF8 (Wijk et al., 2007). The elongation of the primary heart tube, however, is not solely the responsibility of the primary heart-forming regions. Mesodermal cells derived from secondary/ anterior heart fields (SAHF) apparently also contribute to such development (Ramsdell et al., 2005). Such a cell population might be no more than the cranial paraxial mesoderm (CPM) which is known to fill the branchial/pharyngeal arches, together with neural crest (NC) cells, during facial structure formation (Tirosh et al., 2006).

One hypothesis suggests that BMPs from the developing heart tube stimulate cardiac neural crest (CNC) cells. CNC cells are likely to respond to BMPs (such as BMP6 at the OFT) as they contain BMP receptors like Alk2 and Alk3 (Kaartinen et al., 2005). Corroborating with this idea, Kaartinen et al. (2005) has demonstrated that migratory CNC cells co-localize with BMPs and their receptors in mice. In turn, CNC cells signal the SAHF mesodermal cells to migrate to the heart via the activation of cardiac-related genes downstream of BMPs (Kaartinen et al., 2004; Ramsdell et al., 2005; Taimor et al., 2006). When activated, CNCs can enter the heart via 2 distinct pathways to populate the arterial and venus poles via the IFT and the OFT (Poelmann et al., 1999). It is possible that other BMPs are responsible for their distinct patterns of migration.

The differential expression of BMP2 and -4 at stage 10-11 at the inflow and outflow tract, respectively, might control such patterns (Somi et al., 2004). CNC cells do not differentiate into cardiomyocytes and they seem to undergo apoptosis later in development (HH31) (Snider et al., 2007; Poelmann et al., 1999). A possible explanation for such phenomena might be a NC signaling function, as opposed to, a structural function. However, BMP recruitment of neural crest cells and migration of SAHF cells is yet understood. SAHF cells do migrate to the OFT even in stages that CNC cells are not migrating or when specifically ablated (Tirosh et al., 2006).

The fused heart tube will undergo a succession of local constrictions and expansions leading to the formation of chamber-like regions within this hollow tube. The heart is now composed of two major chambers and a rudimentary inflow and outflow tracts (created as a consequence of incomplete fusion at the ends of the heart tube) (Fig. 10). The tube starts with the inflow tract positioned at its inferior side. The inflow tract is composed of the sinus venosus which is the result of the partially confluent left and right sinus horns. Connecting to the sinus horns, and draining blood from the embryo and the yolk, there are two sets of common cardinal veins. Blood entering trough the primitive IFT is drained into the primitive atrium which is separated from the common ventricle by the atrioventricular sulcus (caused by heart tube constriction). The bulbo-ventricular sulcus separates the primitive ventricle from the bulbus cordis (a heart chamber

which will, in part, give rise to the right ventricle). Thus, bulbo-ventricular sulcus can also be called the inter-ventricular sulcus. The remainder part of the bulbus cordis makes the conotruncus which gives rise to the OFT. From the conotruncus the paired dorsal aortae/first aortic arches direct the blood out of the heart tube. The paired dorsal aortae was previously fused to the unfused heart tubes (Larsen, 2003).

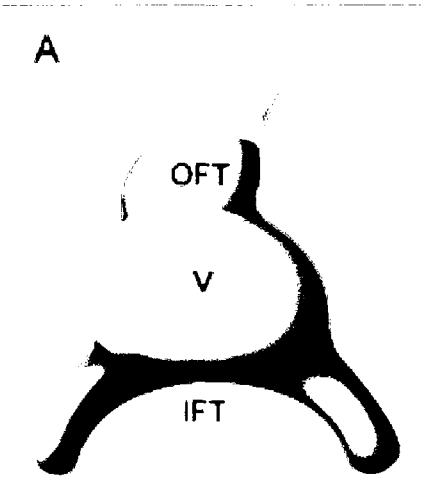

Fig. 10. Schematic diagram of the primitive heart and its chamber-like regions. IFT, inflow tract; OFT, outflow tract; V, ventricle. Reprinted from Wijk et al. (2007), with permission from Oxford University Press.

1.2.3 - Heart Tube Looping & Chamber Formation: Overview.

As a result of a genetic right/left asymmetry system that guides development, the heart tube then bends to the right (dextrally) (Gilbert, 2003;

Levin et al., 1995; Levin et al., 1997; Isaac et al., 1997; Yoshioka et al., 1998; Meno et al., 1998; Ramsdell et al., 2005). With time, the ventricular bend, that resulted from the rightward rotation, shifts toward the atria, creating an S shaped heart (Ramsdell et al., 2005). "The bulbo cordis is displaced inferiorly, ventrally, and to the right; the ventricle is displaced to the left; and the primitive atrium is displaced posteriorly and superiorly" (Larsen, 2003) (Fig. 11). Finally, the distal portion of the OFT moves toward the right atrium. The anterior (arterial) and posterior (venous) poles are brought close together until the heart achieves an atrioventricular concordance (Ramsdell et al., 2005).

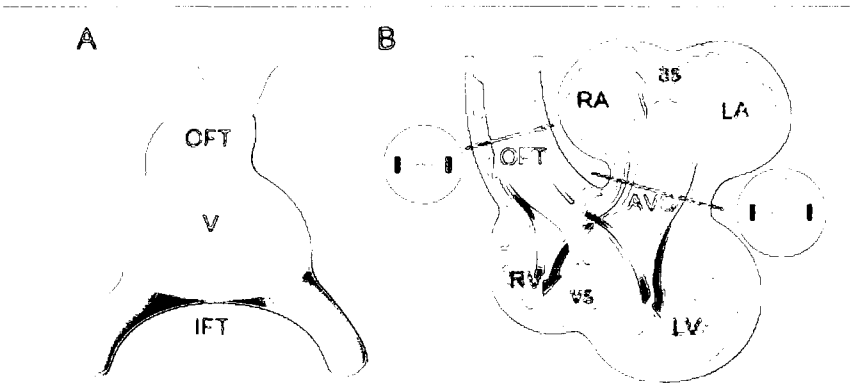

Fig. 11. Schematic diagram of chamber positioning upon heart looping. as, atrial septum; AVC, atrioventricular canal; IFT, inflow tract; LA, left atrium; OFT, outflow tract; RA, right atrium, V, ventricle. Reprinted from Wijk et al. (2007), with permission from Oxford University Press.

Chambers start to form at about HH stage 12 of development in the chick (Wijk et al., 2007) in a process thought to be controlled, at least in part, by BMP2, -4 and -10. At that time (or shortly thereafter) the ventricular wall starts to thicken and begins formation of ventricular trabeculae (Wijk et al., 2007) (Fig. 12). Evidence of the involvement of BMPs includes the presence of BMP10 expression at the sites of chamber formation along the heart tube. BMP10 knockout prevents ventricular formation and is lethal (Wijk et al., 2007). It was found that BMP2 and -4 control the expression of Tbx2, which prevents ventricle extension to the area of the atrioventricular canal (AVC) and OFT (Wijk et al., 2007).

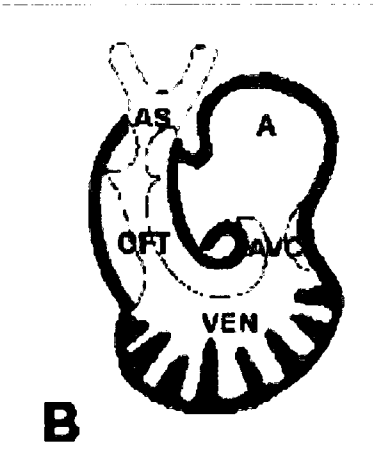

Fig. 12. Schematic diagram of ventricular trabeculation. A, atrium; AS, aortic sac; AVC, atrioventricular canal; OFT, Outflow tract; VEN: ventricle; AVC: atrioventricular canal. Reprinted from Ramsdell et al. (2005), with permission from Elsevier.

Further remodeling will take place to both reshape the OFT and to more correctly position the newly formed chambers. During this remodeling mechanism, the septation of the heart is initiated. This process leads to the division and creation of the final heart chambers.

1.2.4 - Heart Septation: Overview.

Interatrial septation and the formation of endocardial cushions (atrioventricular cushions) starts at around the same time, followed by the formation of the inter-ventricular septum (Larsen, 2003). The alignment of both septa is a consequence of a complex remodeling driven by the continuation of heart looping (Ramsdell et al., 2005). The septation process starts when the roof of the atrium becomes depressed along the superioposterior midline which will evolve into a crecent shaped wedge of tissue called the septum primum (also known as atrial septum primum or ASP) (Jiao et al., 2003; Larsen, 2003). Eventually, the ASP curves to grow toward the back wall causing the atria to become separated but still in communication through a foramen termed ostium primum (Larsen, 2003). When the septum fuses with the growing endocardial cushions, the ostium primum is obliterated. However, the blood continues to flow through the ostium secundum, a foramen, which is the result of the programmed cell death (Larsen, 2003; Jiao et al., 2003). [For a more detailed discussion of intra-atrial wall formation, the reader is directed to Schoenwolf et al., 2009.]

It is believed that soluble signals from the myocardium control the production of the atrioventricular cushions (Larsen, 2003; Lai et al., 2000). These signals cause the endocardial cells to invade the cardiac jelly. The cardiac jelly is the extracellular matrix between the endocardium and myocardium (Jiao et al., 2003). This process is referred to as an epithelial to mesenchymal transformation (EMT), which will lead to the formation of four cushions (superior, inferior, right and left) that grow toward each other (Larsen, 2003). These cushions will eventually fuse together and produce a central mesenchymal mass that will ultimately evolve into the atrioventricular (AV) septum (or septum intermedium) and valves (Jiao et al., 2003; Larsen, 2003).

Septum primum fusion is followed by the formation of the septum secundum (a muscular depression parallel to the atrial septum primum). This septum grows to the back and down but never reaches the endocardial cushions. The space between it and the cushions, termed the foramen ovale, allows blood flow between the atria during development. The baby's first breath at birth results in the opening of the alveoli of the lungs, the opening of the pulmonary circulation, a large drop in peripheral resistance, and an increased pulmonary return to the left atrium. The change in blood volume to the left atrium (and thus pressure in the left atrium) at birth causes the two septa to compress together, forcing the closure of the ostium secundum by the septum secundum and the obliteration of the intra-atrial shunt (Larsen 2003).

This anatomically complex process must clearly demand the spatially and temporally coordinated actions of a number of transcription factors acting on large banks of structural genes. However, the nature of those genes, and when and where they act is still vaguely understood.

1.2.4.1 - The Genes Behind Heart Septation and Valve Formation

As stated previously, beyond a general anatomical understanding, the molecular and cellular process of cushion formation and valvular septation remains poorly characterized and controversial. There appears to be a general consensus, however, that the process seems to require the presence of a variety of members of the TGFß family, including TGFß2 and a number of BMPs. In general, it is believed that BMPs in the myocardium will induce the endocardial epithelium (the inner lining of the heart tube) to transition into a mesenchymal morphology. This process is known as the epithelial to mesenchymal transformation. The mesenchyme, via down regulation of various cellular adhesion molecules, looses its adherence to the endocardial epithelium. It then migrates into the thick layer of extracellular matrix and causes the separation of the endocardium from the myocardium. This mesenchyme accumulates in the primitive atrioventricular canal region (at around HH stage 13) and slightly later-on in the outflow tract. Other cells, predominately if not exclusively neural crest, also migrate to the forming cushions to aid in the ultimate formation of septa and valves (Wijk et al., 2007).

1.2.4.2 - Potential Role for BMP2

Somi et al. (2004) demonstrated the presence of BMP2 transcripts in chicken heart from at least HH stage 16 through stage 24. BMP2 is expressed in both the AV myocardium surrounding the developing cushions and the terminal-most regions of the OFT and the IFT (Fig. 13) (Somi et al., 2004). Similarly, Ma et al. (2005) demonstrated that, in mice, BMP2 is also expressed in the AV myocardium starting at 9.5 days post coitum (DPC). This signal decreases till DPC 12.5 where it becomes confined to the OFT and the ventricular myocardium (Ma et al., 2005).

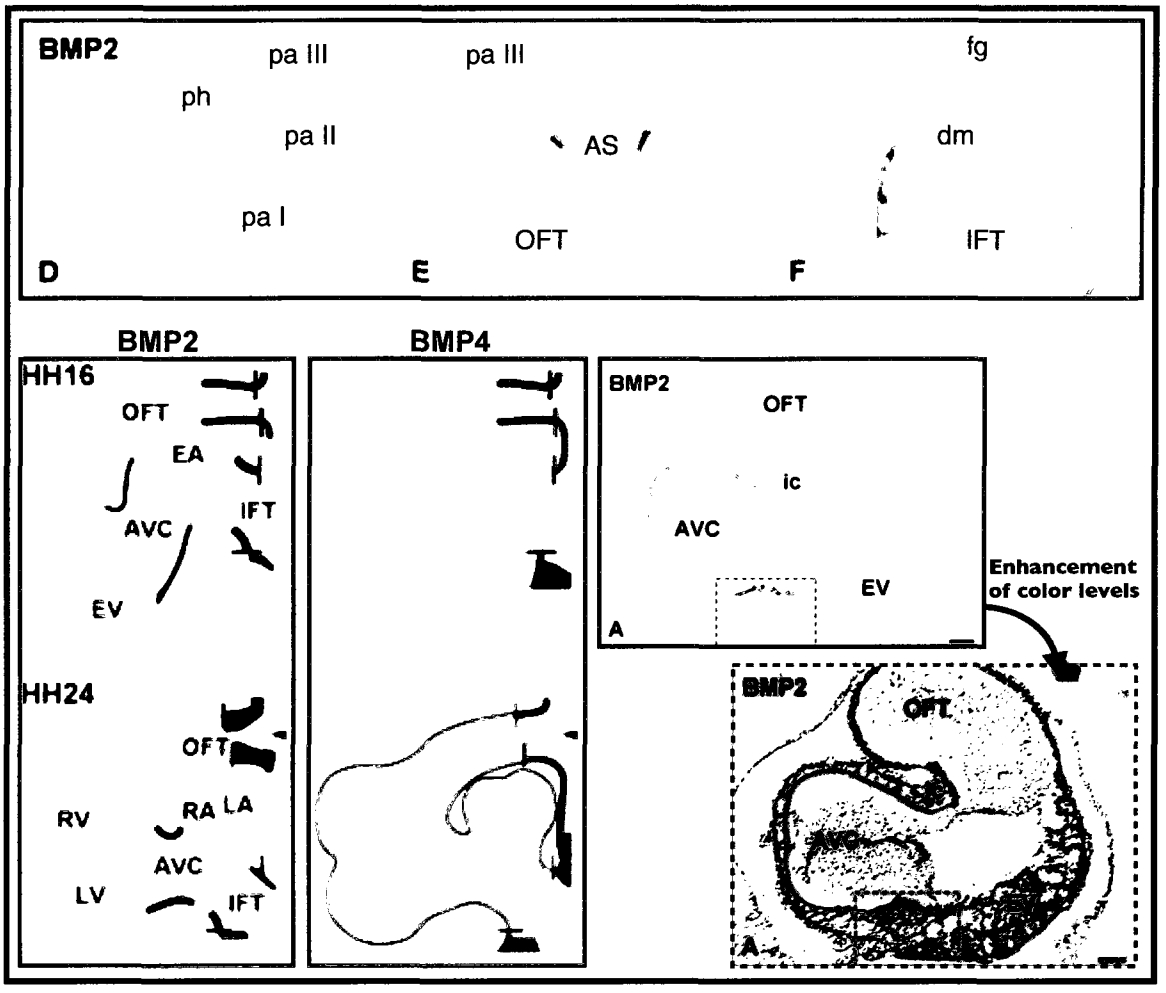

Fig. 13. BMP2 transcription patterns at stage 16-24 (Somi et al., 2004). BMP2 transcripts (*in situ* hybridization) during stage 16 at the pharyngeal archs (D), OFT/aortic sac (E), IFT (F) and cushions at stage 22 (A). Diagrams on the left bottom side panels represent results not demonstrated by pictures. BMP2 but not BMP4 is expressed in the cushion forming regions at both HH16 and 24. AVC, atrioventricular canal; AS, aortic sac; dm, dorsal mesocardium; EA, embryonic atrium; EV, embryonic ventricle; fg, foregut; ic, inner curvature; IFT, inflow tract; LA, left atrium; LV, left ventricle; OFT, outflow tract; pa I-III, pharyngeal arches; ph, pharynx; RA, right atrium; RV, right ventricle. Adapted from Somi et al.

(2004) with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

It is also important to point out that the various BMPs can have distinctly different expression domains within the heart. BMP2, for example, is clearly expressed in the myocardium underlying the cushions from HH stage 16-24, while BMP4 is not (Fig. 14).

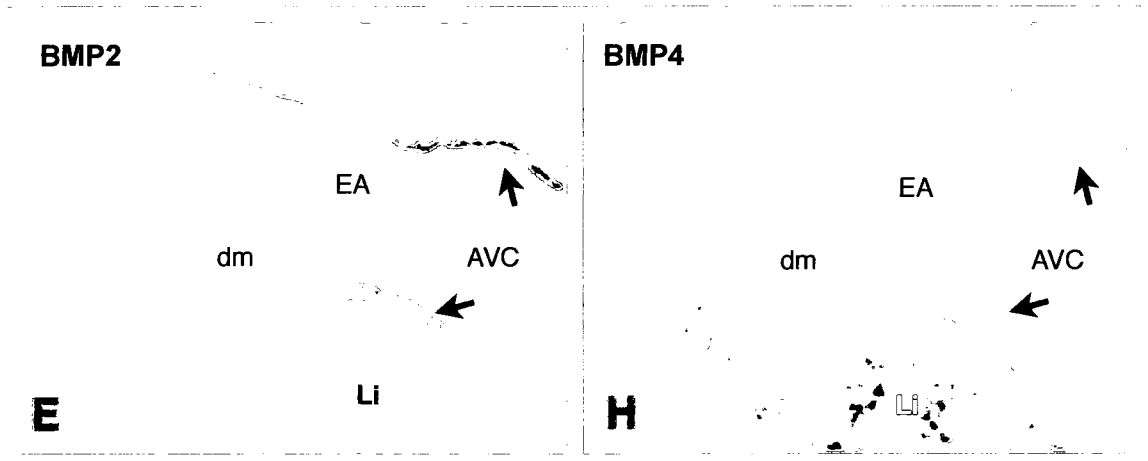

Fig. 14. BMP2 transcripts, but not BMP4, at AVC at HH stage 26 (Somi et al., 2004). MP2 transcripts (E) in the myocardium partially beneath the AVC cushions at stage 23 (see arrows). H: BMP4 transcripts are not detected in association with cushion mesenchyme or related myocardium (see arrows). EA, embryonic atrium; dm, dorsal mesocardium; Li, liver. Adapted from Somi et al. (2004) with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

To further support the hypothesis that BMP2 may play a role in cushion development, Yamada et al. (2000) showed, at HH stage 20-26 of chick development, the presence of BMP2 (and Tbx2) transcripts in tissue surrounding the AVC, as well as at the tip of the inter-ventricular septum (Fig. 15). In contrast, at stage 26, BMP2 seem to populate not only the myocardium beneath the developing cushions (as demonstrated in Fig. 14) but it also populates the mesenchymal cushion themselves (Fig. 15, panel F).

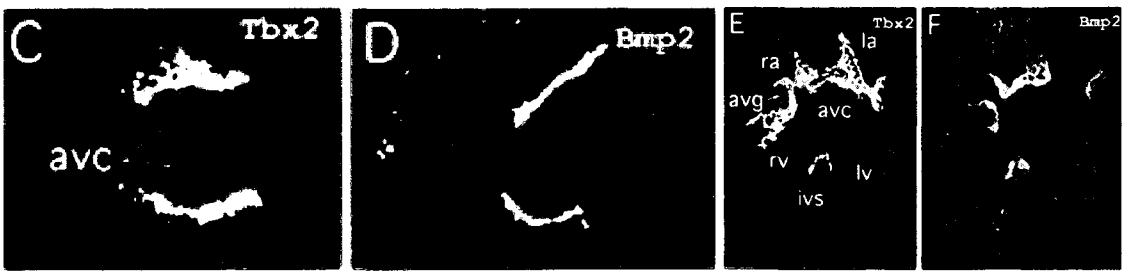

Fig. 15. BMP2 and Tbx2 expression at chicken stage 20-26. Tbx2 and BMP2 expression at: C,D - myocardial expression under the developing cushions; E,F - myocardial expression at the tip of the inter-ventricular and inter-atrial septums, and also in the cushions. AVC, atri-ventricular canal; avg, atri-ventricular groove; ivs, inter-ventricular septum; RV, right ventricle; LV, left ventricle. Reprinted from Yamada et al. (2000), with permission from Elsevier.

Similar to BMP2, Tbx20 is seen to populate the developing cushions at HH stage 18-27, and later the future valves (Yamagishi et al., 2004). These facts, corroborate with the idea previously mentioned that BMPs might indeed activate genes encoding for Tbx2, Tbx20, and possibly others.

There is a considerable body of evidence in supporting the hypothesis that BMP signaling is critical for cushion development. Ma et al. (2005), for example, inactivated BMPr1 (BMPr1 can serve as both a BMP2 or -4 receptor) using a Tie2-Cre transgenic line to show that endocardial BMP1r mutants have no mesenchyme migration. This supports the idea that BMP2 receptors in the endocardium are able to directly interact with myocardially generated BMPs. According to Ma et al. (2005), there is also a direct relationship between BMP2 expressed in the myocardium and the endocardial levels of SMAD proteins (the downstream molecules of the BMP signal transduction cascade). According to Ma et al. (2005), the knockout of BMP2 causes down-regulation of SMADs in the endocardium. To further support the idea that BMP signaling is important in cushion development, cushion formation was found to be SMAD dependent. Desgrosellier et al. (2005) showed that injecting chicken embryos with a SMAD6 adenovirus induced an over-expression of SMAD6 (an inhibitor SMAD or iSMAD) in the AV cushion endocardial cells, which lead to a drastic reduction of cells undergoing EMT. Thus, if endocardial cushions can be inhibited via iSMADs, they are likely to be activated via the BMP pathway.

1.2.4.5 - Possible Roles for BMP4, -5, -6 and -7

In a remarkable series of elegant mouse knockout experiments, Wang et al. (2005) demonstrated that endocardial cells expressing a mutant, defective Alk2 BMP receptor, induced with the use of the Cre system associated to the endocardial promoter Tie2, gave rise to smaller, less well developed cushions (Fig. 16).

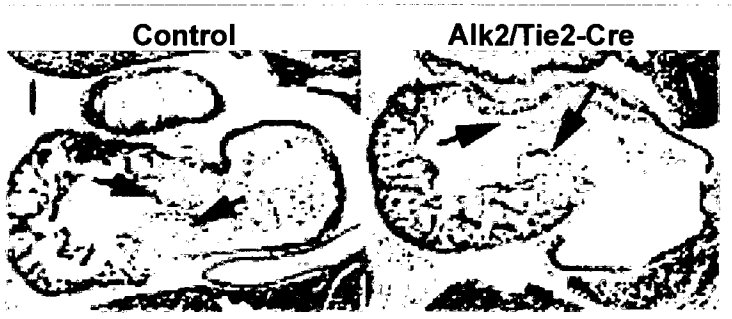

Fig. 16. Alk2 abrogation and cushion malformation. Endocardium specific abrogation of Alk2 expression using the Cre system associated with the endothelial promoter Tie2. Poor cushion development is observed (see space between arrowheads). Arrows point towards endocardial cushions. Reprinted from Wang et al. (2005), with permission from Elsevier.

Since Alk2 is a receptor for BMP5, -6, and -7, it is now believed that these BMPs are also associated with the EMT and the cushion formation (Wang et al., 2005). Somi et al. (2004) showed that both BMP5 and -7 are expressed in various regions of the heart, in patterns that can change significantly as development progresses. Based upon their observations, expression maps for

BMP2, -4, -5, -6 and -7 at various stages of development were created (Fig. 17). At stage HH24, the expression of BMP5, which earlier in development is found throughout the whole heart, exhibits a much more restricted expression domain that localized right beneath the developing cushions (Somi et al., 2004; Wijk et al., 2007; Délot et al., 2003). BMP7, in contrast, is not confined to such regions, but is expressed almost throughout the entire heart. Such expression pattern is maintained until stage 30. It was found that BMP4 expression is confined to the myocardial OFT, partially to the IFT (Fig. 17), and later to the valvular regions (Wijk et al., 2007; Somi et al., 2004). However, BMP4 was not found to populate the AVC (Somi et al., 2004).

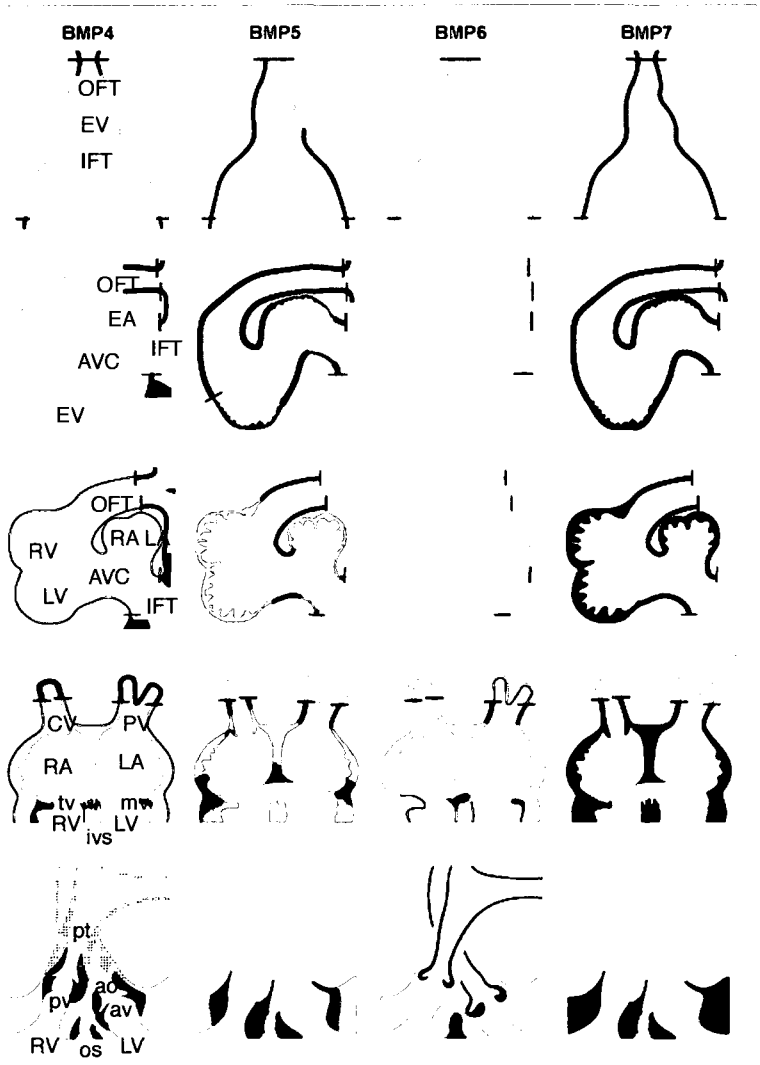

Fig. 17. Schematic diagram of BMP5, -6 and -7 transcription patterns. BMP5 and -7, but not BMP6 is shown to be expressed in the regions of cushion formation. Stages are HH10, 16, 24, 30 and 33 top to bottom. Black represents presence of BMP transcripts for the molecules indicated on top. Light gray represents endocardial cushion tissue. aip, anterior intestinal portal; ao, aorta; av, aortic valve; ea, embryonic atrium; ev, embryonic ventricle; cv, caval vein; IFT, inflow tract; ivs, interventricular septum; LV, left ventricle; LA, left atrium; mv, mitral valve; OFT, outflow tract; os, outlet septum; pt, pulmonary trunk; pv, pulmonary vein; RA, right

atrium; RV, right ventricle; tv, tricuspid valve. Reprinted from Somi et al. (2004), with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

In contrast, studies performed in mice have shown that BMP4 expression exists at a low level in the AVC (Jiao et al., 2003). However, it was demonstrated that BMP4 is not required for the initiation of cushion formation but proper AV septation only occurs in the presence of BMP4 . It is thus likely that BMP4 is required for the progression of cushion development and the formation of atrio-ventricular septa (Jiao et al., 2003).

It was found that in the absence of Alk3, the receptor of BMP2, there is an increased level of myocyte apoptosis which prevents the continuation of cushion development (Gaussin et al. 2002; Schneider et al. 2003). However deletion of BMP4 does not cause myocyte apoptosis. BMP4 is not likely to control myocardium formation or maintenance. Thus, the possibility of the cushion malformation in BMP4 null mutants due to lack of myocytes is excluded. Cushion malformation seems to be the result of an impaired growth of the mesenchymal cells deposited in the cushions (Jiao et al., 2003).

In additional to the atrioventricular septation, BMP4 might also be involved in the valvular development. At stage HH30, BMP4 and -6 are found at the level of the mitral valve (Fig. 18) (Somi et al., 2004).

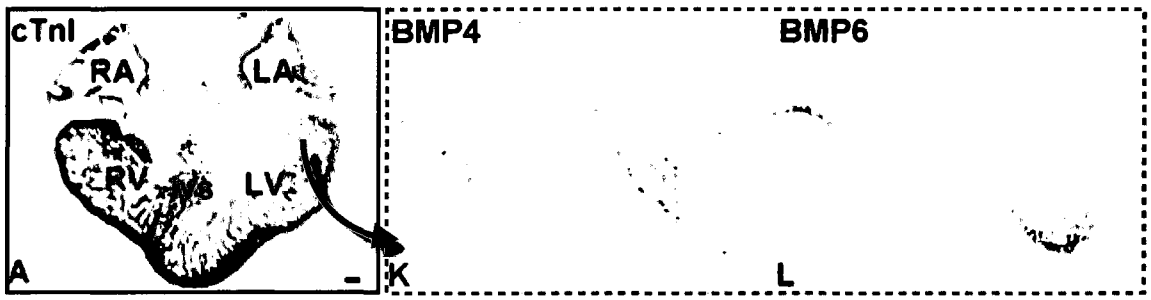

Fig. 18. BMP4 and -6 transcripts at the mitral valve. Ivs, interventricular septum; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. Adapted from Somi et al. (2004), with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

One other striking evidence is that BMP5, -6 and -7 also become confined to the valvular regions at stage HH33 as seen in Fig. 17. Such findings might reveal additional functions of those ligands. One can hypothesize that the major role of BMP6 might be valvular formation, together with BMP4, -5 and -7. BMP6 is not expressed in chick or in mice in the early heart-forming regions (cardiac crescent). In later stages of mouse development, however, BMP6 expression becomes detectable throughout the heart myocardium, including the AV/OFT cushions of the developing heart (Kim et al., 2001; Wijk et al., 2007; Kaartinen et al., 2004). In chicken, BMP6 first appears at HH29 at the OFT outlet septum which develops from the OFT cushions. At HH29, BMP5 is confined to the muscular level around the OFT and AV cushions, and it appears to overlap with the BMP7 domains (Fig. 19).

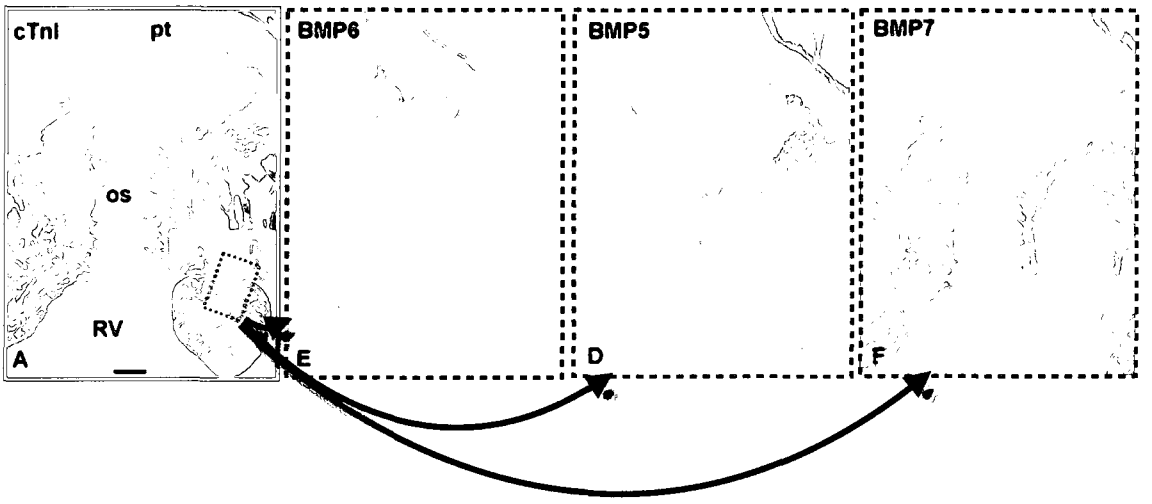

Fig. 19. Transcriptional domains of BMP5, - 6 and -7 at the OFT. BMP5, -6 and -7 transcripts (*in situ* hybridization) at the OFT during stage HH33. Panel "A" shows staining of the entire myocardium using a cardiac troponin mRNA probe and is included to provide anatomical context. Panel "E, D, F" then presents the expression pattern of BMP6, -5 and -7 in the OFT, respectively. BMP5 and -7 partially co-localize at muscular ares of OFT cushions, while BMP6 is seen in the OFT mesenchyme. Adapted from Somi et al. (2004), with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

1.2.4.6 - Relevance of the BMP5, -6 and -7 Receptor Alk2

Alk2 protein receptors are probably the most important type of BMP receptors with regard to cushion development. Alk2 receptors not only seem to

heavily populate the AVC endocardium contributing to EMT, but they also populate neural crest cells, a second cell type that migrates into the heart in response to BMPs expressed in the OFT/IFT (Kaartinen et al., 2004; Ramsdell et al., 2005).

It was suggested that the BMP receptor Alk2 exists with higher abundance in the endocardium when compared to the myocardial levels (Wang et al., 2005; Desgrosellier et al., 2005). This idea corroborates with the notion that BMPs signal from the myocardium to the endocardial receptors, as previously discussed. With respect to cushion development, the mouse endocardial Alk2 receptor appears to be more important than myocardial Alk2 receptor. Cushion formation is normal if Alk2 expression is repressed only in the myocardium using the myocyte specific myocardial MHC promoter (Wang et al., 2005). In contrast, deleting the Alk2 receptors from endocardial cells using a Cre recombinase system and the endocardial cell-specific Tie2 promoter caused detrimental consequences in heart development, including profound atrioseptal defects (Fig. 20).

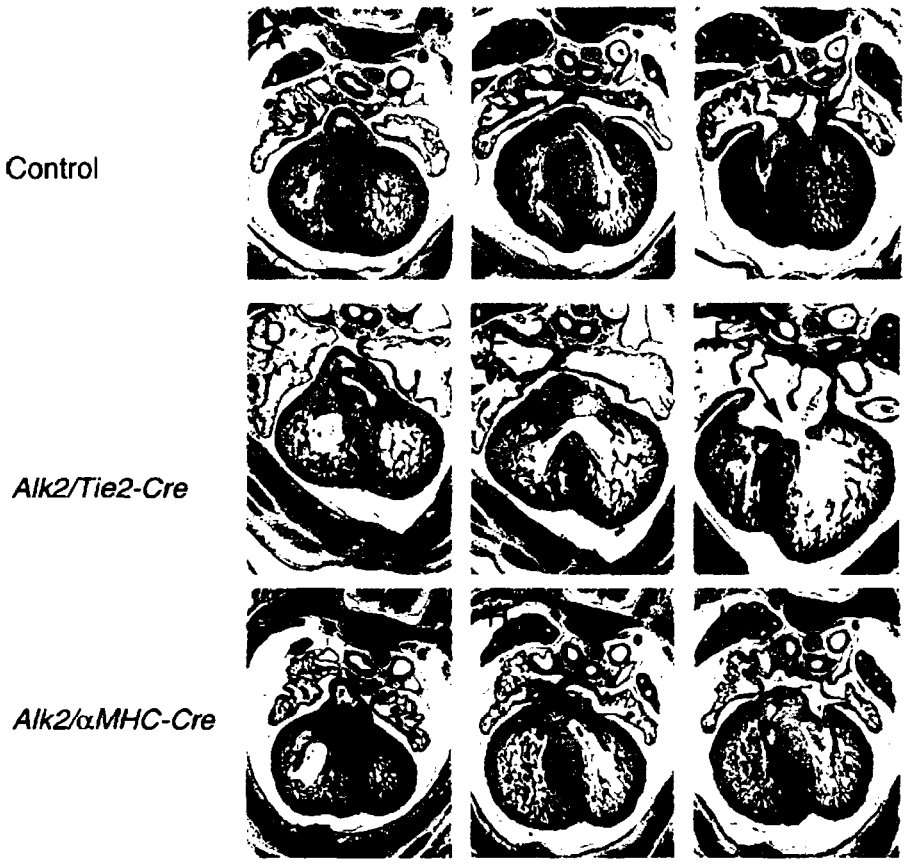

Fig. 20. The contribution of myocardial and endocardial Alk2 receptors to heart development. Abrogation of endocardial Alk2 receptors (D-F) leads to abnormal development of AV cushions (arrows). Abrogation of myocardial Alk2 (G-L) does not appear to impair development when compared to the controls (A-C). Reprinted from Wang et al. (2005), with permission from Elsevier.

The apparent involvement of Alk2 in cushion formation raises the question of whether or not other Alk members also have a role in EMT. Some studies suggested that Alk2 is the most important BMP receptor in mice in the course of

endothelial cellular transformation during EMT (Wang et al., 2005; Desgroselier et al., 2005; Lai et al., 2000). Wang et al. (2005) showed that deletion of Alk2 leads to a decrease in the level of phosphorylated SMAD1, -5, -8 and -2, and thus presumably leads a reduction in their level of activation (Fig. 21).

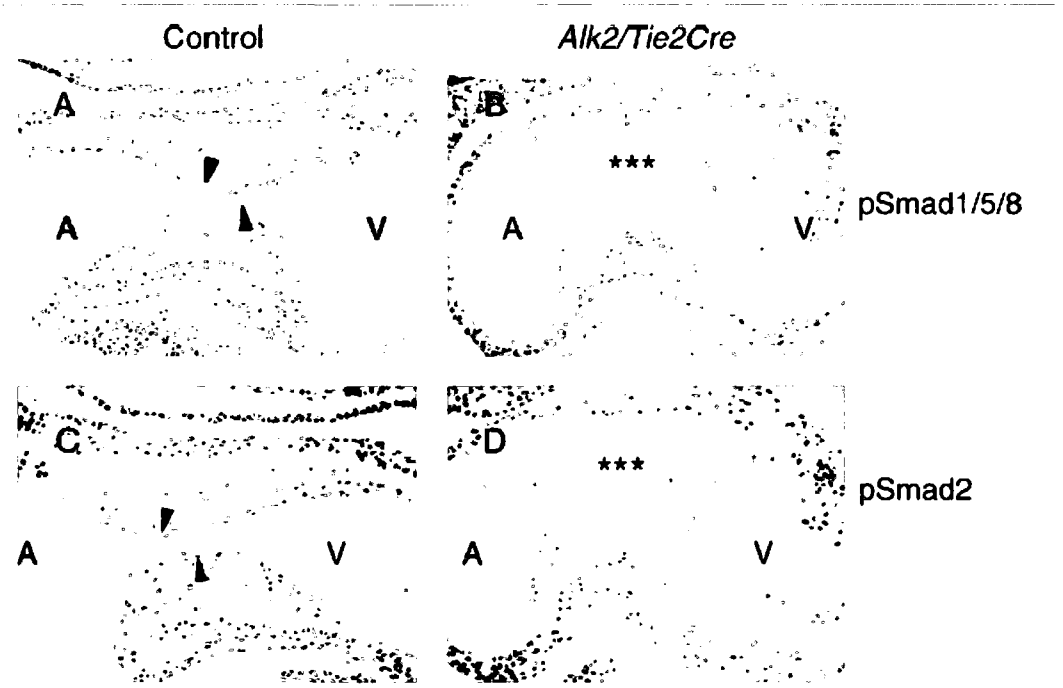

Fig. 21. Alk2 deletion leads to a strong inhibition of SMAD phosphorylation. SMAD phosphorylation was assayed in the control and Alk2 mutants. The use of Tie2Cre system associated to Alk2 leads to a strong reduction of SMAD phosphorylation at the endocardial level (***) when compared to those of the controls (arrowheads). A, atrium; V, ventricle. Reprinted from Wang et al. (2005), with permission from Elsevier.

Despite the fact that Alk1 and -5 seem to work together to respond to the TGFß signals during angiogenesis, their mutations do not seem to affect cushion development (Goumans et al., 2003; Wang et al., 2005). Constitutively active Alk2 appears to induce the ventricular endocardium (cells that normally do not undergo EMT) to transform *in vitro*, while constitutively active Alk5 does not give the same effect (Desgrosellier et al., 2005). Lai et al. (2000) also demonstrated that the repression of Alk2, with an anti-Alk2 specific antibody (Ab), partially inhibits EMT and migration over AV endocardial explants grown in 3D collagen gels. The use of anti-Alk5 Abs has no effect on EMT (Lai et al., 2000).

It is not yet understood how Alk2 might actually mediate EMT. In fact, much of the experimental data suggested that Alk2 might have multiple roles. Wang et al. (2004) clearly showed that cushion development was abnormal in Alk2 knockout mice, and Desgrosellier et al. (2005) showed that Alk2 expression is sufficient to drive at least some degree of ventricular endocardial cell "transformation" *in vitro*. However, it was also demonstrated that *in vivo*, ventricular cells possess Alk2 transcripts, although they do not undergo EMT (Desgrosellier et al., 2005) (Fig. 22). This result could be due to that fact that Alk2 mRNA is not translated in this tissue, or the Alk2 protein does not have the ability to bind to BMPs, or the Alk2 receptor does not activate upon binding of BMPs in the ventricular endocardium. It is also possible that the role of Alk2

receptor is different in various regions of the heart. Clearly, more work in this area is required.

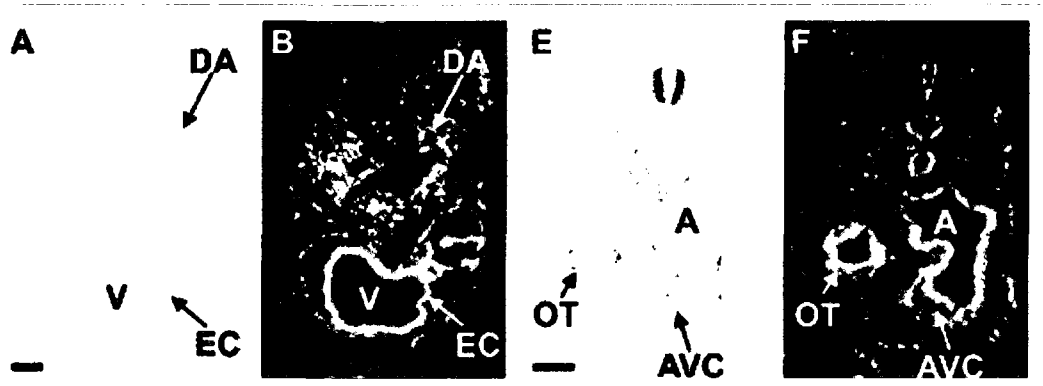

Fig. 22. Endocardial ventricular Alk2. Alk2 mRNA transcripts throughout the endocardium of AV/OFT canals and ventricles (where no cushion formation is known to occur) at stage 14. Red arrows point at the transition between high and low Alk2 transcriptional domains. A, atria; AVC, atrioventricular cushion; EC, endocardium; DA, dorsal aorta; OT, outflow tract; V, ventricle. Reprinted from Desgrosellier et al. (2005), with permission from Elsevier.

Another study suggested that TBRIII, a second class of TGFß receptors which is thought to be solely expressed at the AV/OFT regions, might cooperate with Alk2 in cushion formation (Brown et al., 1999). It was shown that TBRIII expression in endothelial cells that normally do not undergo EMT had undergone EMT and cell migration following expression (Brown et al., 1999) (Fig. 23). Perhaps, TBRIII might be required for the high affinity binding of Alk2 ligands (Brown et al., 1999). This idea might also explain why Desgrosellier et al. (2005)

was able to induce ventricular EMT by over-expressing Alk2. Overexpression of Alk2 might have compensated the low affinity of this receptor for its ligands, in absence of TBRIII.

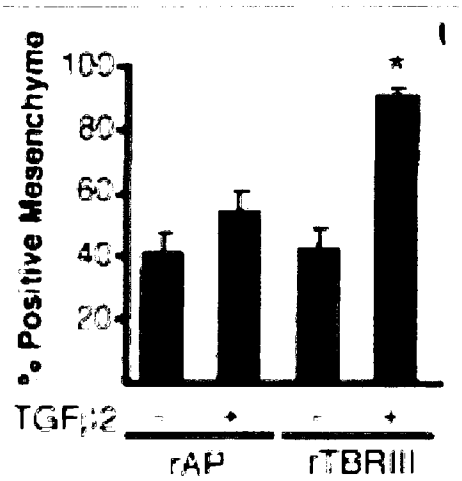

Fig. 23. Ventricular EMT in response to TBRIII. Ventricular explants were grown in presence of TGFß ligands without successful EMT. To test the influence of TBRIII on cellular transformation, it was virally introduced into the cells. Viral targeting and infection consequences were determined via viral infection with alkaline phosphatase. 40% of the cells infected were mesenchyme. Incubation the cells with TGFß ligands did slightly influence the number of mesenchymal cells (an increased percentage observed might be due to the infection of the AV cushions). Infection with TBRIII and incubation with TGFß ligands have an increased effect on the number of migratory cells seen. Support images not shown. Reprinted from Brown et al., 1999 with permission from the American Association for the Advancement of Science.

It is also suggested that TBRI and TBRII are needed to cause AV cushion formation (Brown et al., 1999). TBRIII, due to the small and very localized expression domain in the heart, might act as a regulator of the localization of Alk2 signal transduction (Brown et al., 1999). The association of any of these TBR receptors and Alk2 remains unclear.

Against this backdrop, we have sought to directly map the expression of the Alk2 protein in the developing heart.

2 - LABORATORIAL RESEARCH WORK

2.1 - Overview

It has been postulated that Alk2 receptors might be one of the most important ways of transducing BMP signals during atrioventricular development (Wang et al., 2005; Desgroselier et al., 2005; Lai et al., 2000). Very little information is known, however, regarding the expression patterns of such receptors. Our goal is to determine such expression domains and compare our results to previous experiments. Our approach is a two-step method that starts with the harvest of chicken embryos, their fixation and embedment, and is finally followed by tissue sectioning and treatment of the sections immunohistochemically.

2.2 - Materials and Methods

During the course work performed prior to this thesis a total of seven independent embryo harvesting sessions and many immunohistochemistry (IHC) trials were performed. Slight modifications were made to each protocol in order to improve results. While detailed protocols exist for each modification such changes are only reported in the form of a discussion at the end of this work. The pictures presented in the results section of this thesis are of embryos from harvest 6 (H6) and the procedures we followed are detailed below.

2.2.1 - Tissue Harvesting, Embedment and Fixation

Fertile incubated eggs were obtained from Cal Cruz Hatcheries Inc[1] (Santa Cruz, CA) upon approximately 80 hr incubation at 100 °F. To prevent drastic humidity and temperature changes during transportation, eggs were placed in cardboard egg holders, transported in a styrofoam insulated container, where a bath towel previously soaked in warm water was placed. Upon arrival, the eggs were treated for embryo harvesting as detailed below. In the event that the incubation time of the acquired eggs was inferior than desired, the eggs were incubated in a humidified, temperature controlled, portable incubator equipped with a mechanical rocker system to mediate continuous, slow rocking of the eggs during their incubation period. The eggs were incubated at a constant 37.5 °C while humidity was maintained by daily replenishment of a water reservoir in the incubator.

Harvest was performed using 70% ethanol-sterilized surgical instruments (forceps, scissors and "spoon-type" spatulas), sterile plastic Petri dishes and autoclaved-sterilized calcium/magnesium free Dulbeccos modified phosphate buffered saline (1X-DPBS). Briefly, the eggs were wiped down with 70% ethanol to remove surface bacteria and debri and oriented "air sac up" by placement in a small beaker or bottle cap. Forceps were then used to punch a small hole in the top of the eggshell above the air sac and sections of the eggshell were carefully

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1 **Cal Cruz Hatcheries Inc.** 1010 Rodriguez St, Santa Cruz, CA 95062. Phone: (831) 475-8100

removed to reveal the bottom of the air sac membrane. This membrane was then carefully removed to reveal the embryo, surrounded by the vascularized vitelline membrane (see Fig. 26). Fine surgical scissors were then used to cut the embryo free from the associated vascularized membrane and the embryo was then lifted using a "spoon-type" spatula and transferred to a 35-60 mm Petri dish containing sterile room temperature 1X-DPBS. If there was any significant carry over of egg yolk, the embryos were rinsed in 1X-DPBS and transferred to a second Petri dish prior to further processing.

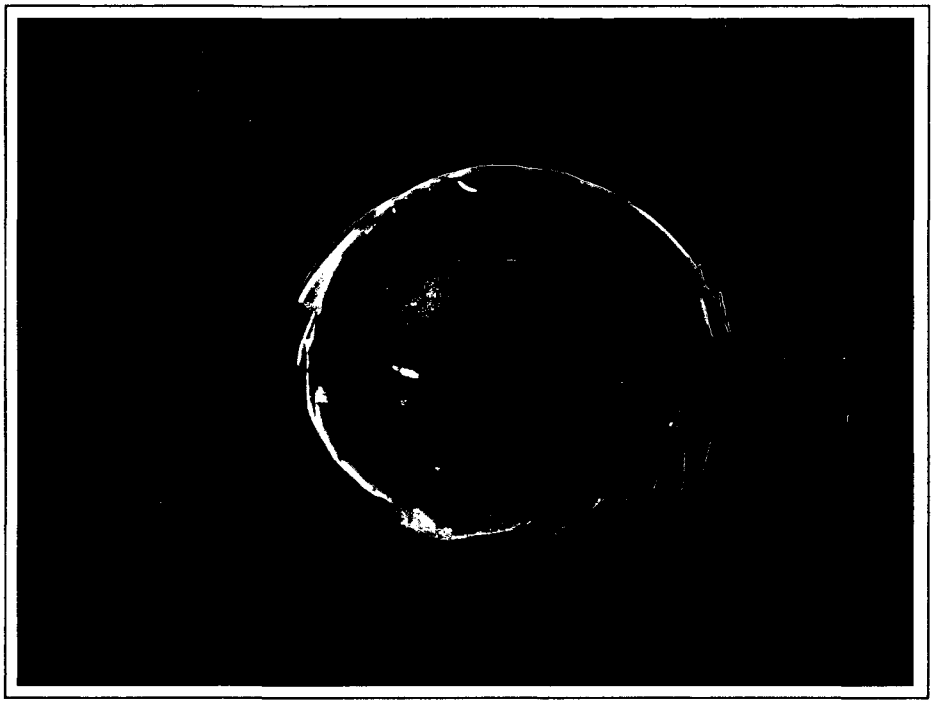

Fig. 24. Embryo harvesting. Demonstration of a developing chicken embryo before isolation. Embryo is connected and surrounded by vascularized vitelline membrane.

At this point any unwanted membranes were trimmed away and the embryo was transferred to a 20 ml glass liquid scintillation counter vial containing approximately 2 ml of cold 4% Paraformaldehyde (PFA) (EMS, 4% Aqueous solution (4% formaldehyde solution), Cat #157-4) to initiate fixation (in general, at least 10 volumes of fixative were added for each one tissue volume). The embryos were then allowed to stand in the fixative for 21 hr at 4 °C.

Fixed tissue was dehydrated at room temperature in a series of ethanol or methanol solutions (25, 50, 70, 85, 95 and 3 times 100%), for 15 min each on a shaker. Such solutions were prepared with Millipore water and either ethanol available in the lab (Rossville Gold Shield Ethyl Alcohol, 200 Proof) or methanol provided by the Biology Department stock room (pure methanol). Embryos one through six were incubated with ethanol-based solutions while embryos seven through twelve were incubated in methanol based solutions. Solutions were carefully decanted between each change. If residual amounts of solutions were left behind, this volume was pipetted using a glass Pasteur pipette pressed against the bottom of the vial. The volume of ethanol/methanol used per vial was approximately the same as that of the discarded fixative. Embryos were incubated 15 min in each solution and Erythrosin B was added to the first 100% alcohol solution to a final concentration of 1mg/ml to permit embryo visualization after embedment. Adding Erythrosin B to one of the later pure alcohol solutions

might lead to the overstaining of the embryos, but we do recommend it for embryos younger than 60 hr of incubation.

After dehydration, embryos were cleared with xylene (EMS, Cat# 23400) by replacing 100% alcohol with a 50/50 alcohol/xylene mixture. The type of alcohol in the mixture depends on the dehydration agent previously used. Solutions were exchanged by decantation. Upon 10 min of incubation, in the alcohol/xylene mixture, tissue was incubated twice in 100% xylene for 10 and 5 min long, respectively. Finally, the embryos were brought to 100% paraffin by replacing xylene with a mixture of xylene/paraffin of increasing concentration (McCormick Scientific, Paraplast Plus, Ref# 502004). First, 100% xylene was decanted and replaced with a 50/50 paraffin/xylene mixture incubated at 60 ºC. After 90 min of incubation in a 60 ºC oven, this solution was replaced with a 75/25 paraffin/xylene mixture and incubated for another 30 min. Finally, this solution was decanted and replaced with melted paraffin. Melted paraffin was replaced a few times to prevent carrying over traces of xylene. Incubations in paraffin were as long as 1 hr, overnight, 4 hr 30 min and 1hr, consecutively. All work containing xylene was performed in a flow hood. All solutions containing paraffin were kept at 60 ºC until the moment of use. This step was performed over a hot plate set for 60 ºC to keep paraffin liquid. Decanting paraffin must be fast to prevent solidification of the solution against the colder areas of the glass vial. Using a pipette to remove leftovers of paraffin is sometimes necessary.

Paraffin will solidify promptly inside the pipette so one must place the pipette inside a melted paraffin vial placed over the hot plate when not in use.

After incubation in paraffin, embryos were casted into thin aluminum circular molding trays approximately 5 cm in diameter. Trays were placed over a 60 °C hot plate containing a thin layer of melted paraffin. Paraffin blocks were identified with the harvest number ID (H6) and the type of alcohol used during dehydration by a small piece of paper placed on the inner side of the molding tray rim. Vials containing the embryos were inverted into the tray and the embryos were distributed equally spaced, 5mm away from the tray's periphery with 5 embryos per tray. In order to completely cover all embryos, more paraffin was added as necessary. Trays were carefully lifted off the hot plate and placed on the bench top for solidification. Once solid, trays were placed over distilled water (diH2O) with paraffin side up and flipped after 5 min using a small beaker as weight for a more complete solidification. The resultant casts were kept at 4 °C until use.

Aluminum trays were separated from the paraffin block, which was sequentially scored with a razor blade. The block was then broken in half by applying pressure on each side of the score. Individual blocks of one embryo each were obtained by this method and reshaped into a parallelepiped using a razor blade. Approximated tissue orientation relative to the faces of the parallelepiped was achieved during trimming of such geometrical shape by

carefully placing each embryo closer to one of its smaller faces. A hot spatula was placed over a rotary microtome wood mounting block and the smaller face of the created block was placed over the hot spatula. It is important that the orientation of the paraffin block is done in such a way that the embryo side of the paraffin block is placed farther away from the wood block. Once enough paraffin has melted (equivalent to 1-2mm of the base of the block) knife was removed and paraffin left to solidify. For a better adhesion of the paraffin to the wood block, paraffin from each side of the parallelepiped (except the side closer to the embryo, the one previously in contact with the aluminum tray) was melted with the aid of an hot spatula. Once complete solidification was achieved at 4 ºC the paraffin block was trimmed with a razor blade to minimize the amount of paraffin surrounding the embryo. No more that 1-2mm of paraffin is needed on each side. For a visual depiction of such methodology refer to the DVD annexed to this thesis (supplement 6).

The blocks were then mounted on an American Optical Corporation Spencer "820" manual rotary microtome with mechanical advance. 10µm sections were obtained using a sharp microtome knife (American Optical Corporation). Sections were obtained as ribbons (a result of the pressure that one section exerts over the previously cut section at the edge of the blade). These long strips were then taken off the knife (using a scalpel) and placed over a clean flat surface. The ribbon was cut to small pieces and lifted using a spatula

containing a tinny water droplet at its edge. Ribbons were placed floating in a 40 ºC water bath to straighten and mounted on Fisherbrand* Superfrost* Excell* microscope slides (Fisher Co., Cat# 22-034-985) as described next. Correctly labeled microscope slides were immersed in water with its charged surface side up. Using a spatula or needle, the paraffin and the slide were brought together. Once the first section has adhered to the slide, the slide was gently pulled up from the water in a perpendicular position. Slides were allowed to dry vertically at room temperature for 1-2 hr. Fig. 25 represents a Superfrost Excell slide with adherent paraffin sections ready for IHC.

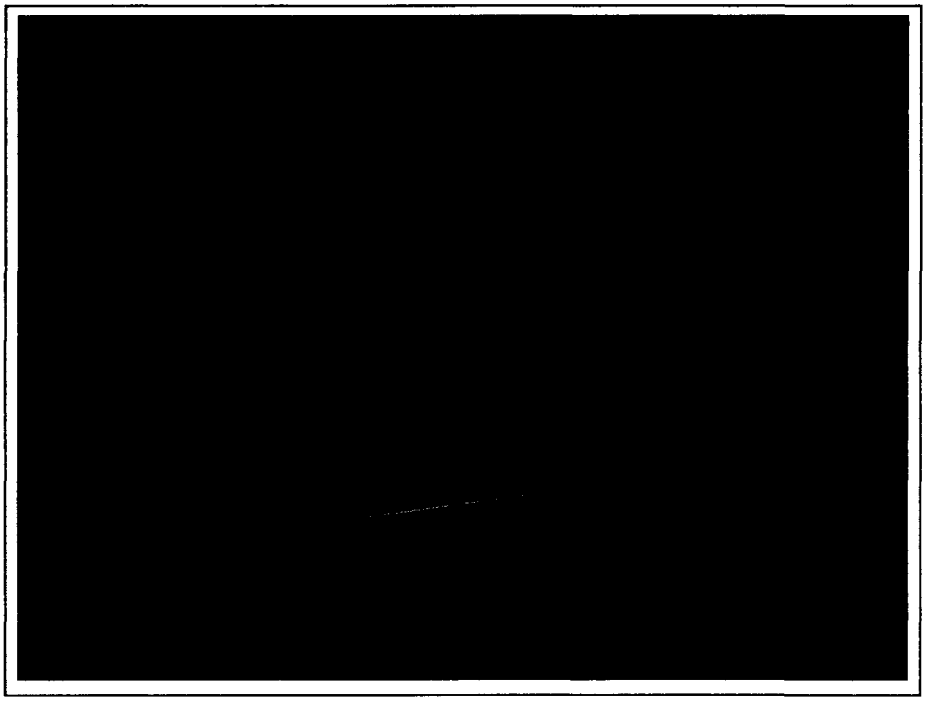

Fig. 25. Fisherbrand* Superfrost* Excell* microscope slide with paraffin-embedded tissue sections. Pink represents embryonic tissue stained with Erythrosin B.

Sections were allowed to dry for 2 hr at room temperature followed by overnight incubation at 37 °C slanted at 45-60° horizontally relatively to the surface (Fig. 26).

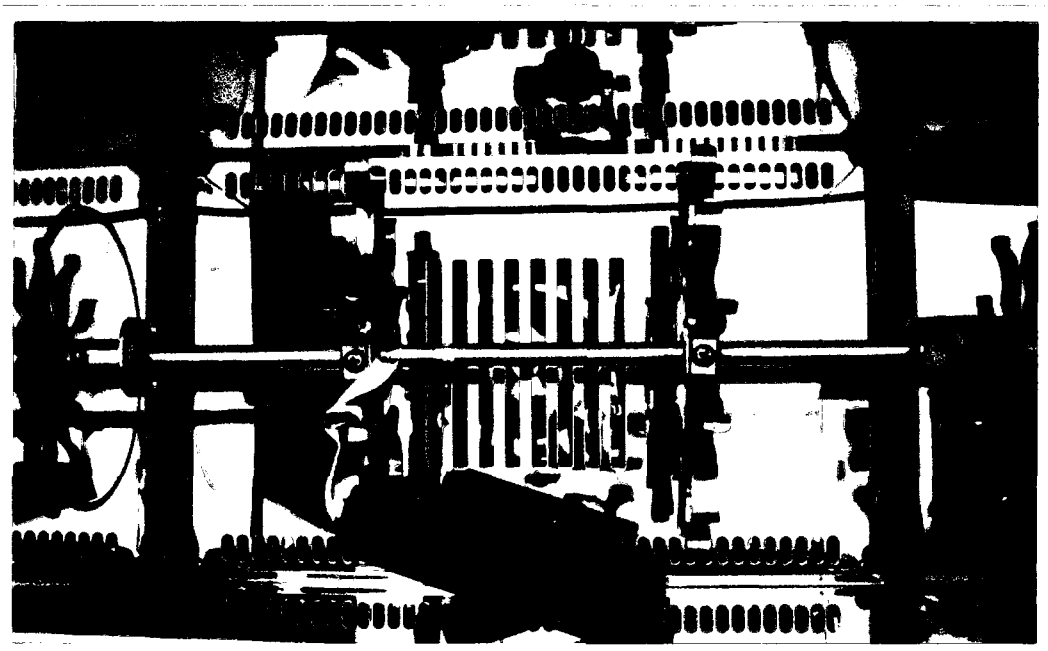

Fig. 26. Baking of sectioned tissue. Recently (1-2 h) section tissue was baked at 37 °C at a slight angle.

See supplement 6 for a audio-visual protocol of the treatment of casted paraffin embedded embryos, provided in form of VideoCD.

2.2.2 - Immunohistochemistry

Embedded tissue sections were deparaffinized by immersion in 3 changes of xylene, 5 min each in the fume hood (xylene was placed in standard Coplin jars containing approximately 50 ml of solution). Following deparafinization, the mounted tissue was rehydrated by passing the slides through a series of ethanol solutions (100% > 95% > 85% > 50% > 25%, using Pharmco - Reagent Alcohol / 200 proof, Ethanol 90.25%, Methanol 4.75%, IPA 5%; Cat# 241000200). Slides were immersed in each alcohol solution for 3 min each to facilitate gradual rehydration. Finally, the slides were immersed in 2 changes of distilled water, 3 min per change, to remove any trace of alcohol.

Slides were removed individually from the water filled Coplin jars. Quickly, the surface surrounding the tissue sections was cleaned with the edges of a folded kimwipe for maximal absorption and dexterity to prevent dehydration. A hydrophobic barrier was quickly drawn around the tissue sections using Vector Laboratories, ImmEdge Hydrophobic Barrier Pen, Cat# H-4000. Sections were then flooded with PBS/BSA (PBA) for blocking (common used ratio: 50µl for 5 tissue section) by gently pipetting the solution to one of the inner edges of the hydrophobic barrier. PBA was prepared by diluting Sigma Albumin from bovine serum, Cat# A7906 to 1% w/v in sterile 1X-DPBS. *Other blocking solution could be used.* PBA was kept frozen in small aliquots and defrosted as needed. Slides

were then incubated in a 37 °C incubator for 1 hr in a humid chamber (Petri dish containing drops of water and slides elevated above the water).

The blocking solution was then tapped off by placing the slide vertically with the long side against a kimwipe. A gentle movement is enough for most liquid covering the sections to defeat the hydrophobic barrier and slide down to the kimwipe. Each section was then covered with approximately 10µl of primary Ab that was previously diluted in aPBA (PBS/acetylated BSA). The Ab solution should not be pipetted directly over the tissue sections to prevent damage. aPBA was prepared by diluting Promega - R396D - Bovine Serum Albumin Acetylated, 10mg/ml, 100x dilution required, using sterile 1X-DPBS, aliquoted in sterile micro-centrifuge tubes and frozen until use. After allowed to thaw, aPBA was kept refrigerated. While the duration of incubation with the primary Ab typically requires some optimization, a 1 hr and 20 min incubation at 37 °C is enough for some Abs. Primary Abs used in these studies are goat anti-mouse Alk2 (Santa Cruz Biotechnology's ACTR-I (F14) Cat# sc-31295, diluted 1:200 in PBS/acetylated BSA) and anti-chicken notochord (Hybridoma Bank, Cat# 15.3B9[NOT1][2], diluted 1:5 in PBS/acetylated BSA) as a positive control. Incubations were performed in a humidified chamber as described above to prevent desiccation and Ab precipitation.

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2 The monoclonal antibody developed by Jessell, T.M. and Dodd, J. was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Primary Ab was removed by tapping it off against a kimwipe. Any residual volume that did not slide over the hydrophobic barrier was then absorbed using the edge of a folded kimwipe. Residual unbounded Ab was washed off by flooding the sections with PBS twice for 5 min each.

Similar to what was done when using primary Ab, slides were then covered with secondary Ab and incubated for 1 hr at 37 °C in a humid chamber. Secondary Abs used in this study were donkey anti-goat IgG-AP (Santa Cruz Biotechnology's Cat# sc-2022) and donkey anti-mouse IgG-AP (Santa Cruz Biotechnology's Cat# sc-2316) both diluted to a concentration of 1:2000 in aPBA.

Following the incubation in the secondary Abs, slides were rinsed in PBS as mentioned above, and then covered with 5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium (BCIP/NBT) (SIGMA FAST™ BCIP/NBT, Sigma-Aldrich Biotechnology LP, Cat# B5655) (aprox. 50µl per 5 sections). Incubation was performed at room temperature until the desired color was observed without excessive BCIP/NBT precipitation. This was achieved in approximately 15min, but no longer than 25. Color development was quenched by immersing the slide in distilled water filled Coplin jar 2 to 3 times.

Tissue sections were then dehydrated by immersing the slides in a series of increasing concentration of 200-proof ethanol solutions (95% > 100% > 100%). Slides were dipped 4 times in each solution followed by four dips in 1:1 ethanol:xylene and 100% xylene. Slides were left in 100% xylene a little longer

until no Brownian movements were seen. When using a five-slide gripper to hold more than one slide at a time reagents are often carried-over from one solution to another. Avoid immersing the gripper into the solutions and if water is carried over to the xylenes let the slides rest until all water has deposited on the bottom of the jar. Slides were retrieved from xylene and one or two droplets of permount were added to the sections. A cover slip was placed at an angle over the sections to allow the permount to diffuse and cover all tissue. Slides were then allowed to dry overnight.

2.3 - Results

Previous work has demonstrated that Alk2 is likely one of the most important BMP receptors expressed during heart septation. Null mutants for Alk2 have highly decreased levels of active SMAD proteins and defective cushion formation (Wang et al., 2005). Very little information, however, has been presented on the patterns of ACTR-I transcription and expression. One of the only attempts to demonstrate the domains of Alk2 transcription was done by Desgrosellier and colleagues (2005) who were able to demonstrate the presence of Alk2 mRNA in the AV/OFT and ventricular endocardium, a novel and somewhat unexpected result. Except for inter-ventricular septation, no cushion formation is known to occur in the ventricle. While Alk2 receptors might propagate differential signals when expressed in different cells (in the AV/OFT *vs.*

ventricular region) it is also possible that mRNA translation is abrogated and its expression functionally silenced in the ventricles by a mechanism yet unknown. To make matters even more difficult, the Desgrosellier study has never been repeated or the reported Alk2 expression domain confirmed in any way. Our study tries to resolve these ambiguities by examining the Alk2 protein expression patterns directly via IHC. Even with significant tissue degradation that was observed during our immunohistochemical approach (which was unavoidable despite all the efforts to improve tissue preservation) we have determined novel expression patterns for Alk2 receptors. At stage 4 of development (80 hr of incubation) Alk2 is expressed on the atrium wall and through the growing septum primum (which at this time serves to partially divide the right and left atrium) (Fig. 27 panel A). As we get closer to the undivided atrium, the Alk2 protein expression domain changes to become expressed in two main domains, one right beneath the developing endocardial cushions (EC) and the second scattered around the outflow tract (OFT)/Conus arteriosus (Fig. 27 panel B). When a second embryo (emrbyo #8, represented as H6-8) from the same harvest was independently treated for IHC similar results were observed (Fig. 28, 29). In the caudal direction, the conus arteriosus (OFT) meets and fuses with the heart. Scattered Alk2 domains are also found in the trabeculated ventricle (Fig. 27 panel D) in concordance with Desgrosellier's study. However, such expression domains are only observed in a very restricted number of ventricular sections (see Fig. 27 panels C through E and Fig. 30 panels D and E). Note that

these results were also observed in at least two independently treated embryos from the same harvest and of similar age (Harvest 6 embryo 9 (Fig. 27) and embryo 8 (Fig. 30)). The images presented in the following pages are accompanied by schematic diagrams of the heart shape and section level. Such diagrams have been created to give the reader an approximate idea of each section's depth and orientation relative to major heart structures (OFT *vs.* atrium, proximity to septum primum/ ceiling of the atrium *vs.* endocardial cushions, etc.) and should not be interpreted as a perfect representation of the heart.

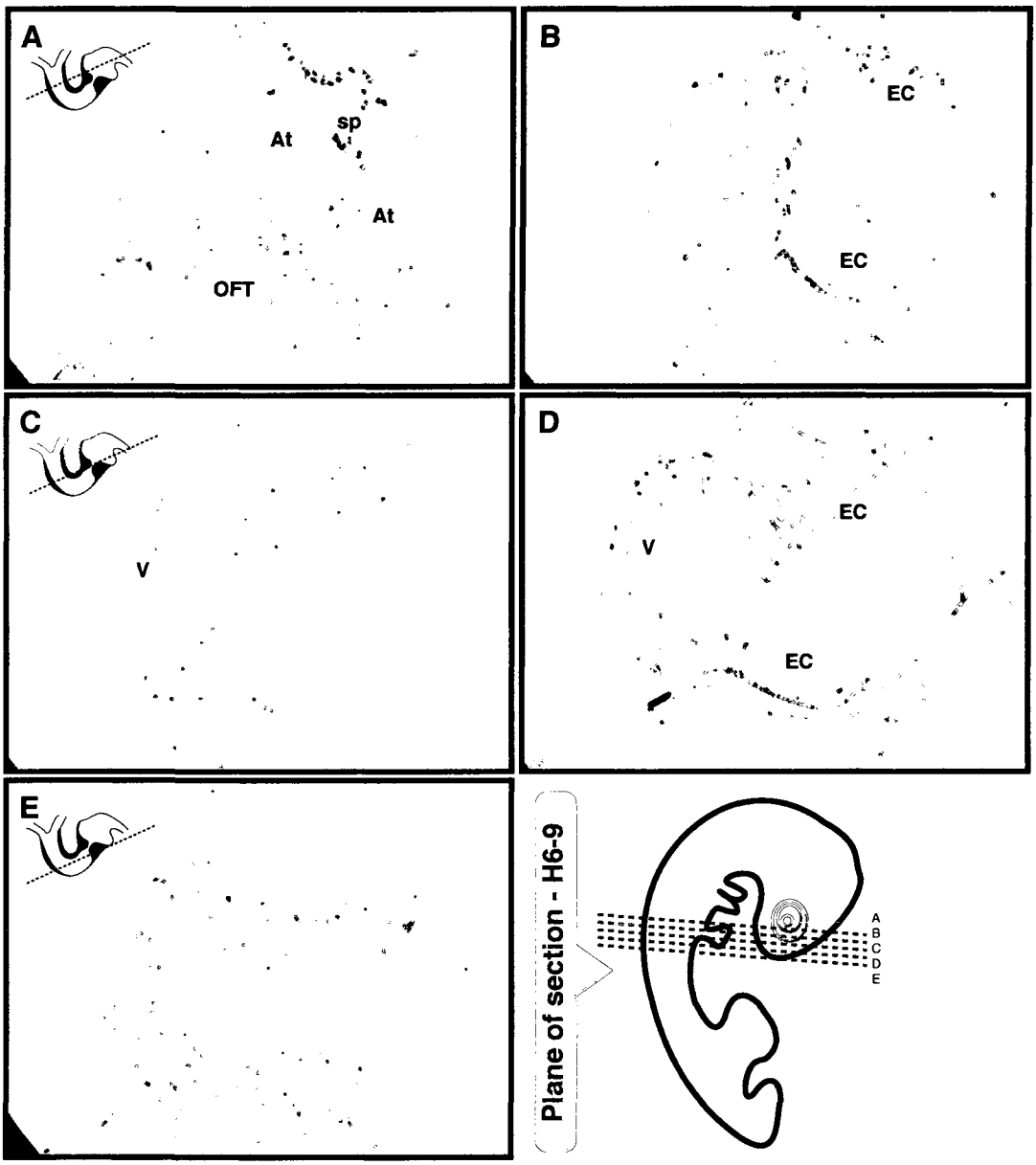

Fig. 27. Expression of cardiac Alk2 at embryonic day 4 (embryo H6-9). Transversal sections through the chick embryonic heart at day four. Sections (from embryo H6-9) were incubated with 1:200 anti-Alk2 Ab and 1:2000 secondary Ab. BCIP development occurred on atrial walls (atrium = at) surrounding the endocardial cushions (EC) and scattered along the trabeculated ventricle (v). The septum primum (sp) which divides the right

and left atrium is also populated by Alk2 (Panel A). Pictures were post-processed on Adobe Photoshop CS4 to dodge shadows created by the camera on the field of view edges.

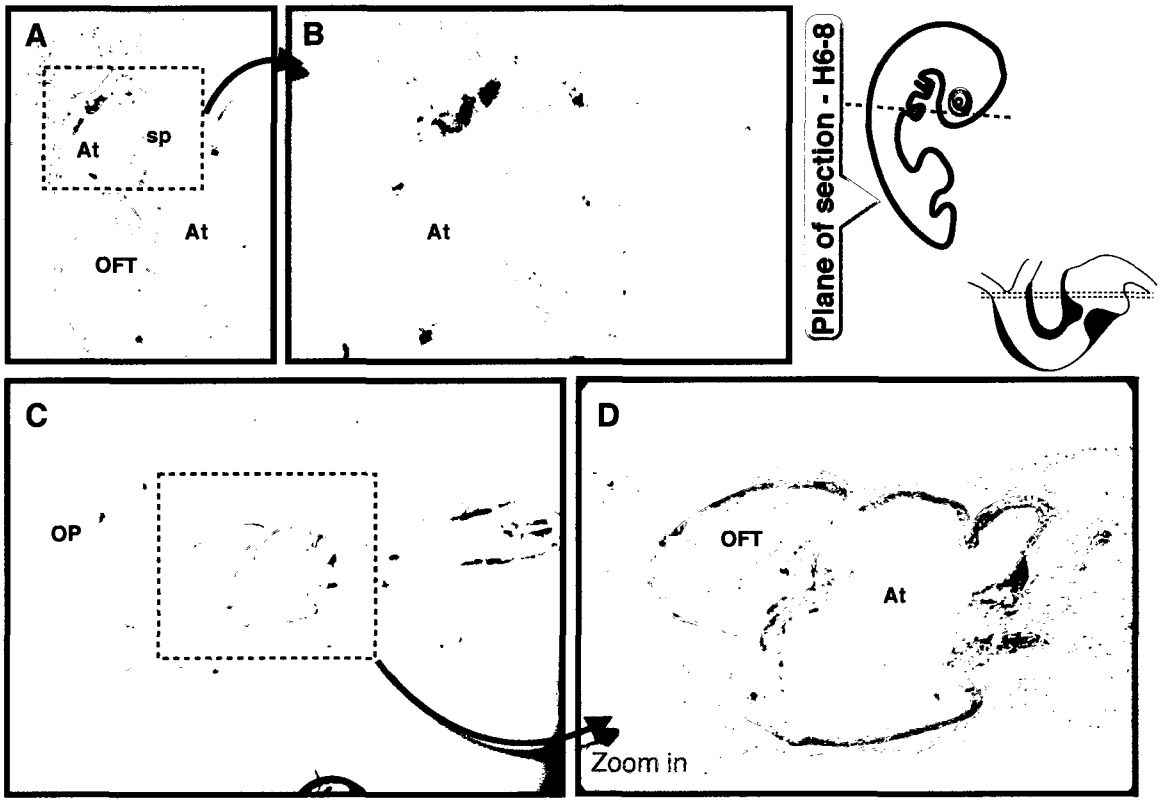

Fig. 28. Expression of cardiac Alk2 at embryonic day 4 (embryo H6-8): the atrium and septum primum. Two transversal sections through the chick embryonic heart at day four are shown above. Sections (from embryo H6-8) were incubated with 1:200 anti-Alk2 Ab and 1:2000 secondary Ab. BCIP development occurred on the base of the endocardial cushions (EC) and scattered along the outflow tract (OFT). The septum primum (sp) partially dividing the left and right atrium is also populated by Alk2. Optical Pit (OP) has been labeled for anatomical reference. Pictures were post-

processed on Adobe Photoshop CS4 to dodge shadows created by the camera on the field of view edges.

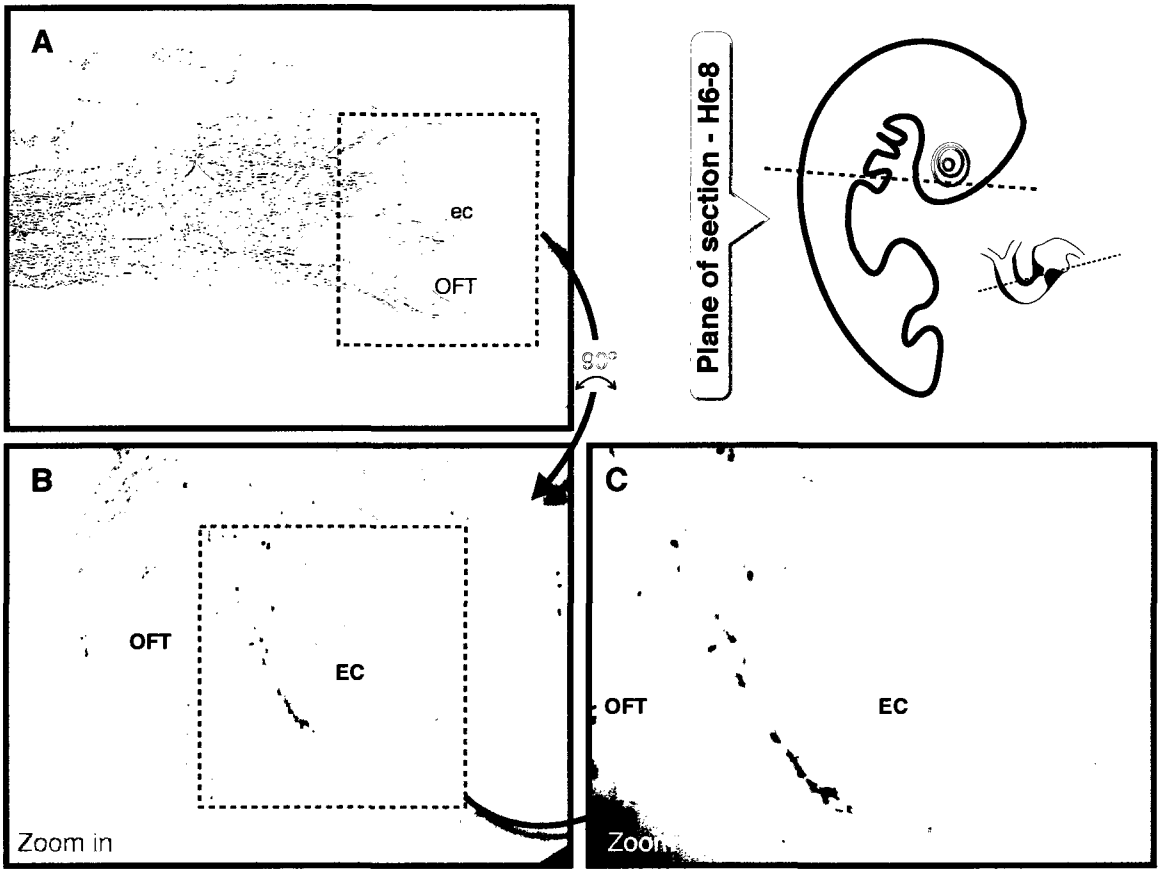

Fig. 29. Expression of cardiac Alk2 at embryonic day 4 (embryo H6-8): the endocardial cushions. A transversal section through the chick embryonic heart at day four is shown above. Sections (from embryo H6-8) were incubated with 1:200 anti-Alk2 Ab and 1:2000 secondary Ab. BCIP development occurred on the base of the endocardial cushions (EC) in a similar pattern to those observed in Fig. 30 panel B. Pictures were post-processed on Adobe Photoshop CS4 to dodge shadows created by the camera on the field of view edges.

Emrbyo H6-8 gave us a clearer view of the patterns of expression of Alk2 in the ventricle. As depicted in Fig. 30, the ventricle also appears to be populated by Alk2, however, such an expression pattern seems to be confined to only a few set of ventricular sections. Panel D, demonstrates that sections posterior to those seen in panel C are absent of any Alk2 signal.

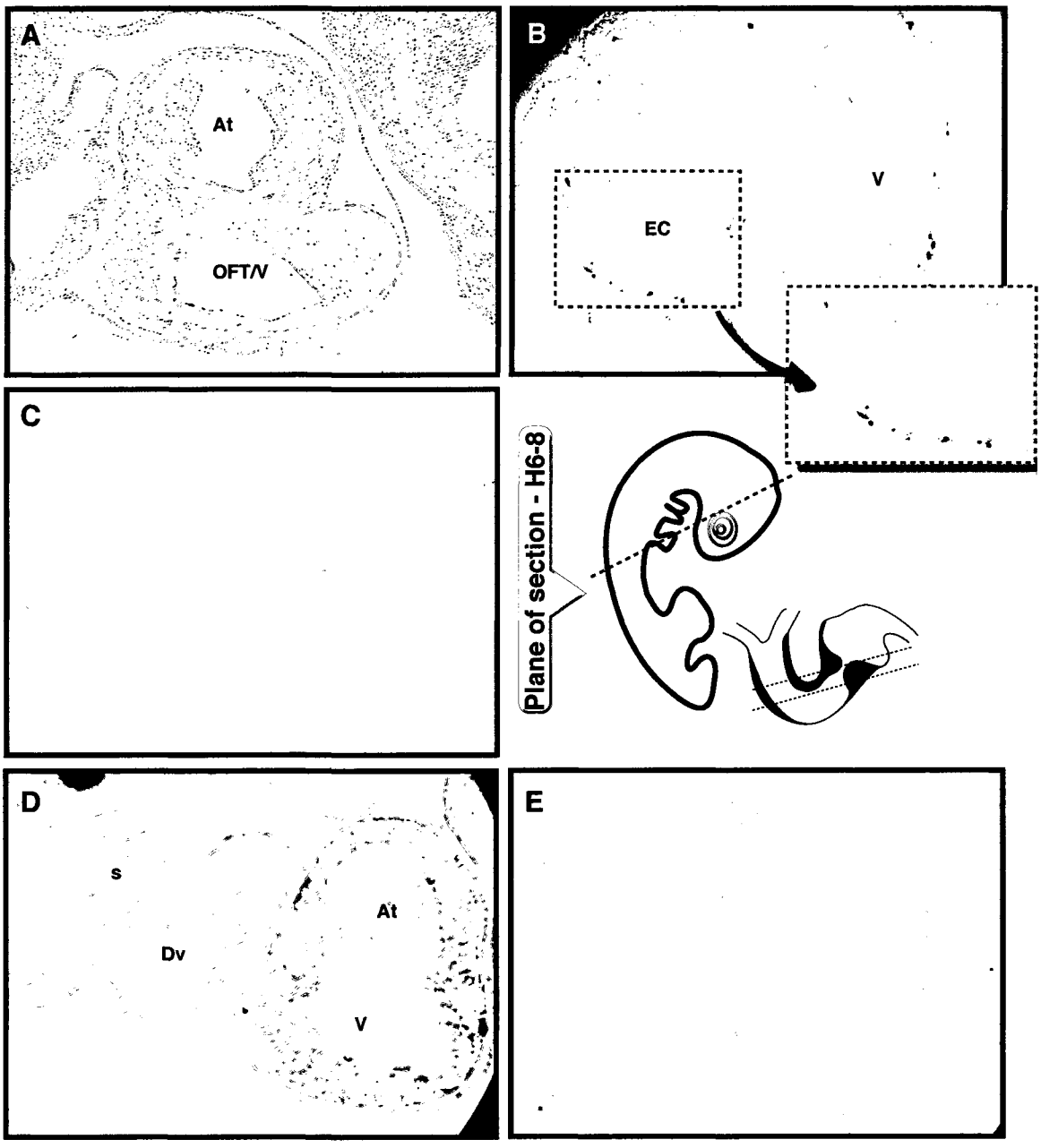

Fig. 30. Expression of cardiac Alk2 at embryonic day 4 (embryo H6-8): the EC cushions and ventricle. Transversal sections through the chick embryonic heart at day four are shown above. Sections (from embryo H6-8) were incubated with 1:200 anti-Alk2 Ab and 1:2000 secondary Ab. BCIP development occurred on the base of the endocardial cushions (EC)

and along the trabeculated ventricle (v). Ventricular expression of Alk2 seems to be restricted to a few set of ventricular sections (compare D and E). For orientation, ductos venosus (Dv), stomach (s) and outflow tract/ ventricle (OFT/V) were labeled. Pictures were post-processed on Adobe Photoshop CS4 to dodge shadows created by the camera on the field of view edges.

The results obtained were heart-specific as indicated by the absence of signal in nearby tissues. Fig. 31 shows a magnified section of the digestive system (esophagus/trachea region) where no signal is observed.

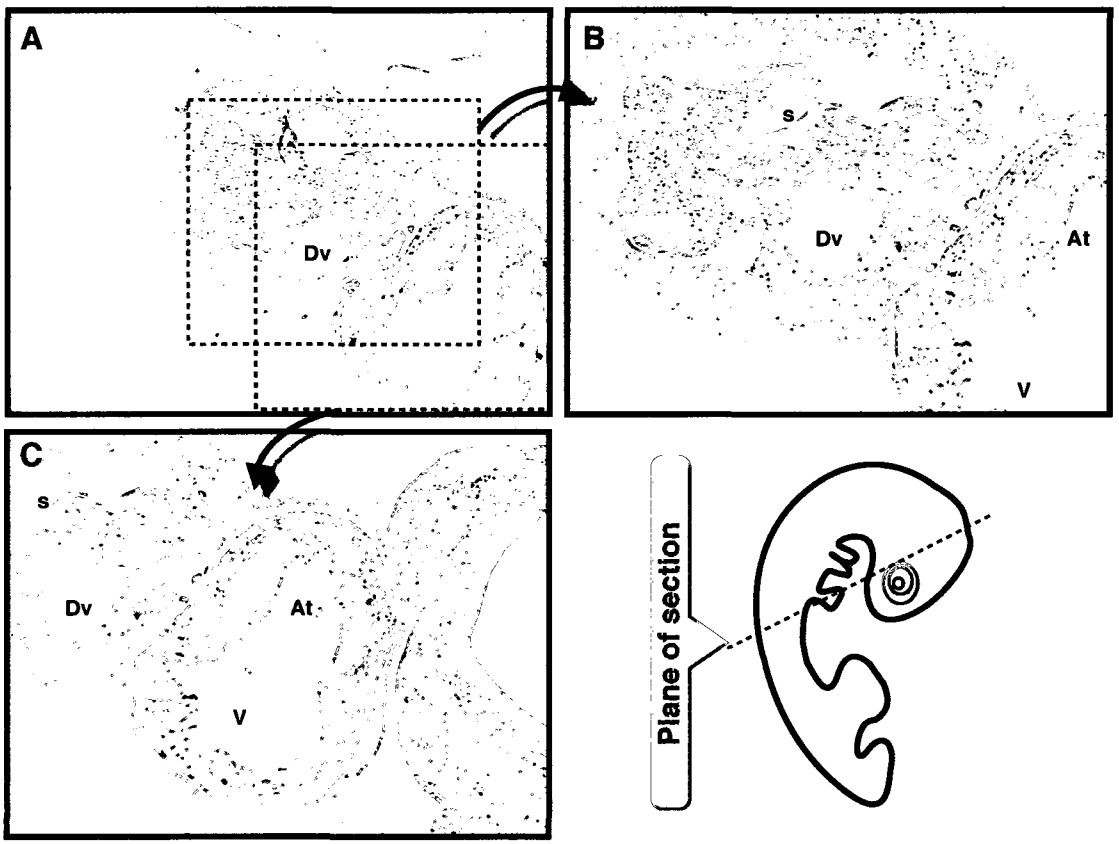

Fig. 31. Absence of non specific signals on treated sections (embryo H6-8). A transverse section through the chick embryonic heart at day four is shown above. Sections (from embryo H6-8) were incubated with 1:200 Alk2 Ab and 1:2000 secondary Ab. Panel B and C do not derive from panel A but they are of close nearby sections. Stomach (s) and ductus venosus (Dv) were labeled for orientation.

The staining results of the section derived from embryo H6-4 corroborate with Fig. 27 and 29 (panels A and B) where it was demonstrated that Alk2 receptors not only populate the atrial walls but also the descending septum primum. In the next few figures (Fig. 32, panels B through E) one can observe such results from a slightly different angle that cuts through the atrium upwards toward the descending septum primum.

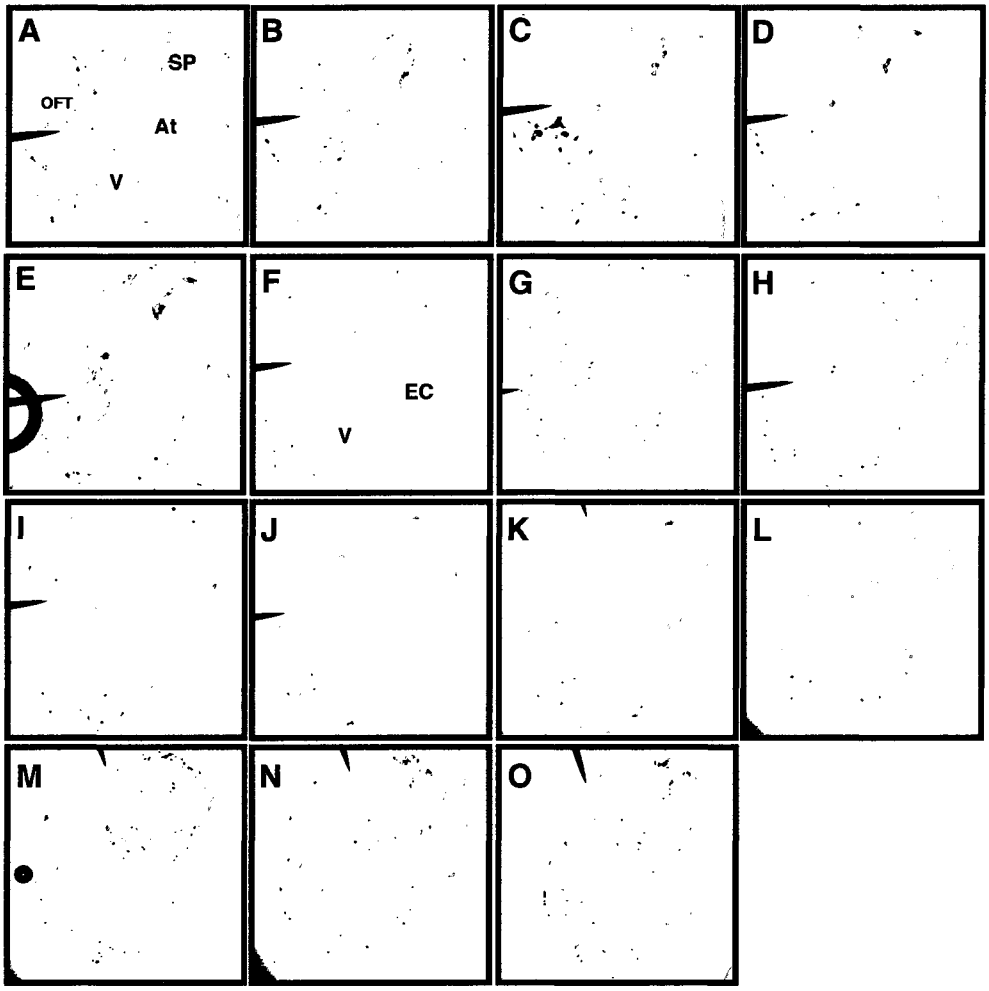

Fig. 32. Alk2 and the septum primum (embryo H6-4). Panel A through O show serial sections of the heart in a semi-transversal plane. The atrium (at), being invaded by descending septum primum (SP) can be seen. The concentration of Alk2 seems to increase in the septum primum from image A to E. In addition, the expression domain of Alk2 in these images is comparable with those from Fig. 27 & 29 panel A and B. In panel J the septum primum meets and fuses with the growing endocardial cushions dividing the right and left atria. The difference in size between the two atria is likely related to the plane of section. In panel M the two

endocardial cushions meet and fuse causing a physical but partial separation of the atria and ventricle.

2.4 - Discussion

Careful analyses of the presented results seem to indicate that Alk2 protein expression might indeed be related to endocardial cushion development. In this study, the Alk2 protein can be detected on the walls of the atrium (in and around the septum primum and the endocardial cushions). What is curious when comparing our results with those discussed by Desgrosellier et al. (2005) and the studies of Wang et al. (2005) is the fact that the domains of Alk2 expression found in this study are primarily myocardial/epicardial. Studies by Desgrosellier et al. (2005), suggest that Alk2 mRNA transcripts exist throughout the embryo but, with respect to the heart, are expressed in the endocardium of AV/OFT canals, in the AV cushions, and in the ventricle (Fig. 33).

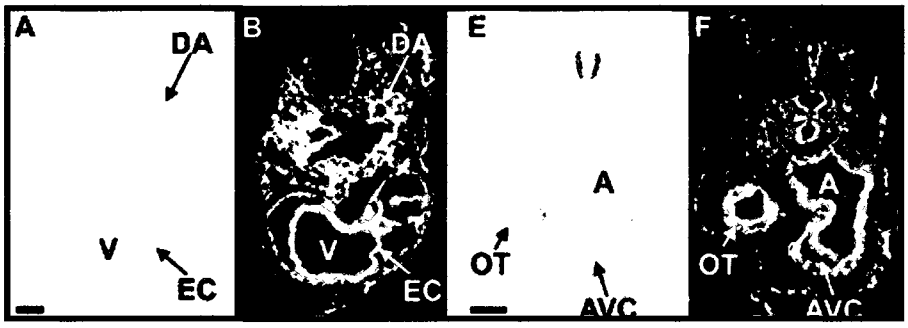

Fig. 33. Endocardial Alk2 transcripts in the developing chicken heart. Alk2 mRNA transcripts throughout the endocardium of AV/OFT canals and ventricles (where no cushion formation is known to occur) of an stage 14

embryo (Hamburger Hamilton Staging. Stage 14 = 50-53 hr of development). V, ventricle; EC, endocardium; DA, dorsal aorta; OT, outflow tract; A, atria; AVC. atrioventricular cushion. Reprinted from Desgrosellier et al. (2005), with permission from Elsevier.

Fig. 34 provides an expanded view of the results obtained both by us and by Desgrosellier. While the left panel (Desgrosellier's results) shows Alk2 mRNA being expressed in the endocardial lining of the cushions, the panel on the right (our results) demonstrates something quite different. Our results show the Alk2 protein at the outer edge of the myocardial layer or the epicardium. The fact that we find Alk2 protein expression in regions of the heart where Alk2 mRNA is not known to exist raises several questions. Adding to the controversy are the results reported by Wang et al. (2005). According to Wang, inhibition of myocardial Alk2 expression has no effect on atrioventricular cushion formation and shape, while inhibition of endocardial Alk2 expression greatly diminishes cushion formation. Despite these conflicting data reports, our protein localization results were repeatable at least once (in different sections of the same embryo and between two different embryos), suggesting that they are not an experimental artifact (Fig. 35).

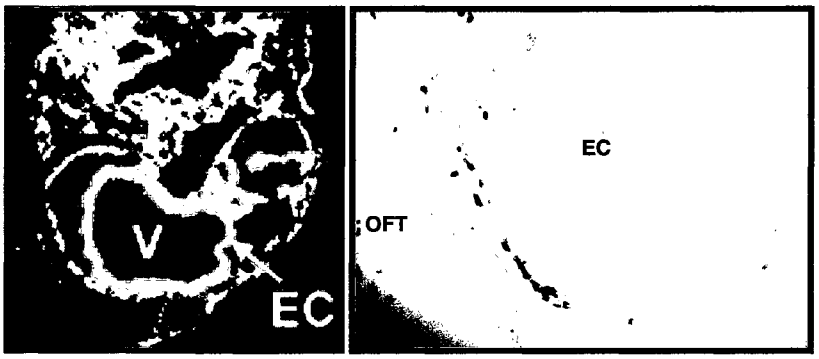

Fig. 34. Endocardial vs. myocardial Alk2 expression. The panel on the left demonstrates the results obtained by Desgrosellier and colleagues (2005) where Alk2 mRNA is seen to be present lining the inner curvature (in relationship with the inside of the heart) of the endocardial cushions (EC). The panel on the right depicts the myocardial expression of the Alk2 protein found in this study. V, ventricle; OFT, outflow tract. Left panel was weprinted from Desgrosellier et al. (2005), with permission from Elsevier.

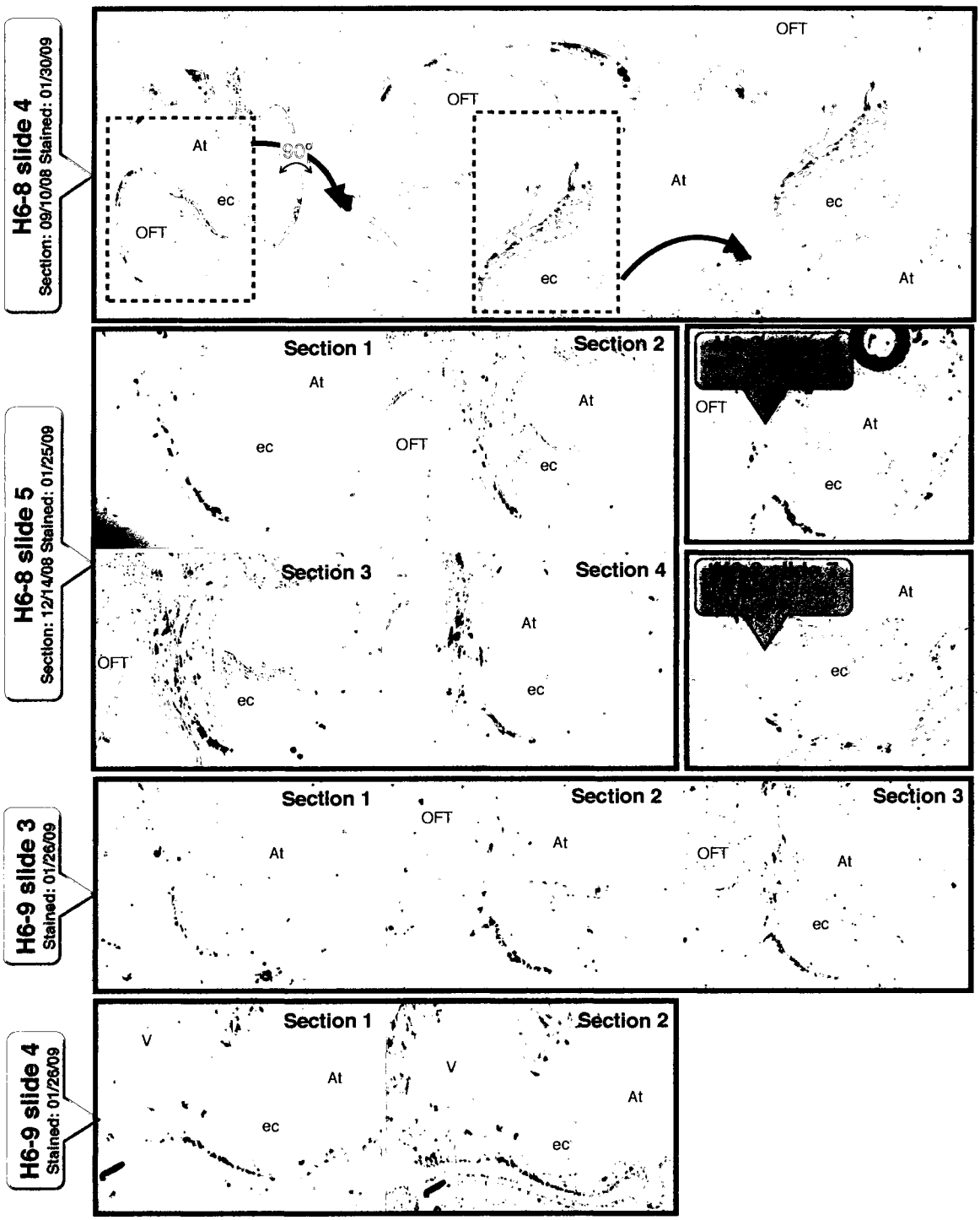

Fig. 35. Myocardial/epicardial expression of Alk2: repeatability. Expression of Alk2 in the myocardial/endocardial layer underlying the

endocardial cushions (ec). To demonstrate reproducibility two different embryos (#8 and 9) from harvest 6 were studied (H6-8 / H6-9). Sections of each embryo were divided into different slides and processed independently in different days using an anti-Alk2 antibody (see materials and methods). H6-8 slide 5 and 7 were stained on 01/25/09. H6-8 slide 4 and 6 were stained on 01/30/09. H6-9 slide 3 and 4 were stained at 01/26/09. One or more sections from each slide are represented. Myocardial/epicardial staining of Alk2 is depicted in blue. At, atrium; ec, endocardial cushions; OFT, outflow tract; V, ventricle. Sharpness of each picture was increased for better visualization of tissue.

One possible explanation to account for these different findings is that Alk2 expression might be subject to a very specific spatial and temporal control. While the function of endocardial Alk2 might be the induction of EMT, myocardial and/or epicardial Alk2 might play important roles at later stages of development. Thus, it may be possible that Desgrosellier only detected Alk2 mRNA in the endocardium because he studied slightly different stages of development. During development, even small differences in age might lead to drastic changes in the genetic expression patterns. It is possible that Alk2 mRNA, and then Alk2 protein expression is activated in the endocardium or myocardium/epicardium at a different developmental time point.

Technical issues related to experimental procedures might also contribute to the explanation of the observed discrepancies. In our experiments, the use of

paraformaldehyde-fixed embryos might suppress signal detection due to cross-linking and masking that can randomly occur over the population of epitopes. Similarly, cross-linking and masking might also provide a basis for the uneven, "punctate" (dot-like) staining pattern we observe in many of our sections. Unless the tissue is over-fixed, however, some proteins will always have native (unfixed, not cross-linked) epitopes. Assuming that these epitopes are accessible and in a sufficiently high local concentration across the surface of a population of Alk2 proteins, they might then allow detection without the necessity of exposing the tissue to a (generally damaging) "antigen retrieval" protocol. It is possible then, that at the stage of development under study in our experiments, a low expression level of endocardial Alk2 proteins combined with fixation-related epitope masking, prevents the detection of Alk2. In light of these considerations, we recommend that this study be repeated with implementation of a shorter Ag retrieval step, with the hope of optimizing the signal.

Differences in expression patterns between experiments might also occur if Alk2 exists in multiple protein isoforms that vary in their expression patterns between the endocardial and myocardial/epicardial tissues. This could explain the findings if the mRNA probe used by Desgrosellier detects an endocardial isoform while the antibody we used (raised against an epitope not disclosed to us by the manufacturer) detects a different myocardial/epicardial isoform that arises from a splice variant. One way of testing this theory is to produce RNA probes

spanning the entire length of non-mature unspliced mRNA. When used in an *in situ* hybridization such probes would likely allow the detection of all possible different splice variants that would give rise to all possible protein isoforms. This technique could be used to look for Alk2 RNA expression in the myocardial/ epicardial tissues where we detect the Alk2 protein. If nothing else, a bioinformatics approach to determine the likelihood of existence of possible isoforms for Alk2 protein must be performed. Regardless, whether our results are pure artifact or a novel discovery, further work must be done to elucidate the involvement of Alk2 in heart development.

Despite the disagreement between our work and that of Desgrosellier et al. with regard to expression in the endocardium *vs.* myo-or epicardium, it is important to point out that the regional localization of Alk2 receptors described in this thesis still generally coincides with the transcription patterns of BMP5 and -7 (known ligands of Alk2 receptors) (Somi et al., 2004) (Fig. 36).

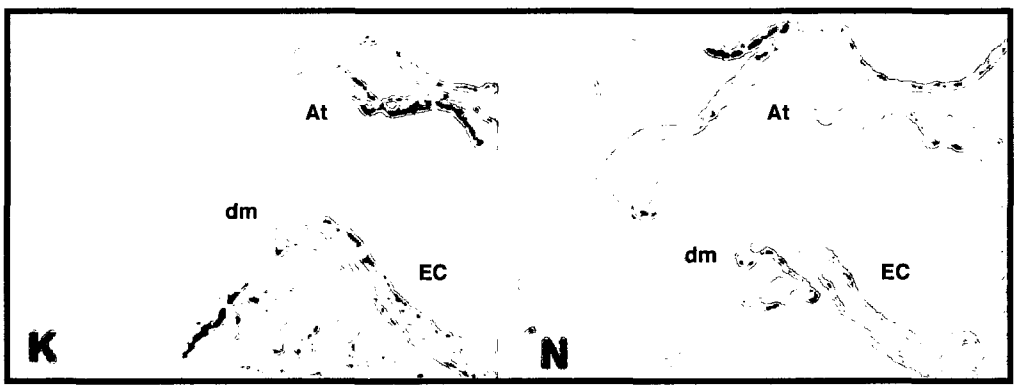

Fig. 36. Cushion development: BMP5 and -7 transcripts. BMP5 (panel K) and BMP7 (panel N) transcripts are seen in the AVC myocardium right

beneath the developing cushions of a stage 23 embryo (aprox. 80 hr of development). Photo contrast was enhanced so that cushions are visible. At, atrium; Ec, endocardial cushions; dm, dorsal mesocardium. Reprinted from Somi et al. (2004) with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

Somi, has not only demonstrated that BMP5 is present right beneath the developing cushions but that it also populates the OFT, again, in general agreement with the patterns of Alk2 expression found in this study. The next figure (Fig. 37) is a schematic summary of Somi's results.

How our results relate with those of Somi in the context of cushion development is not well understood. As discussed above we demonstrate that Alk2 expression seems to be confined to the outer myocardial and/or epicardial layer, which is hard to reconcile with the idea that BMPs exclusively generated in the myocardium diffuse through the cardiac jelly, signal endocardial cells to become mesenchyme, and ultimately drive those cells to loose adherence to the endocardial epithelium as they commit to EMT transformation.

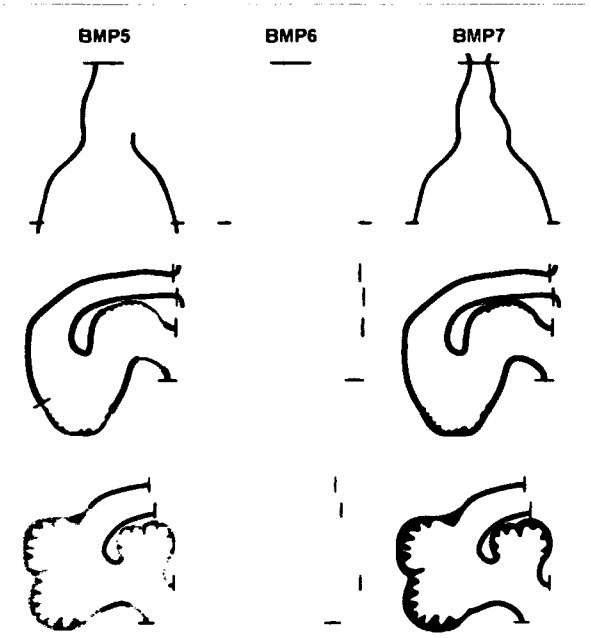

Fig. 37. Schematic diagram of BM5, -6 and -7 transcription patterns. BMP5 and -7, but not BMP6 are expressed in the regions of cushion formation. Stages are HH10, -16, -24 top to bottom. Reprinted from Somi et al. (2004) with permission from Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.

One other aspect of importance to be discussed in this thesis is the complexity and sensitivity of the technical work performed. Immunohistochemistry is a complex technique where success depends on many factors. From the moment of tissue harvest to the final moment of Ab detection, every step has crucial consequences for the stability and availability of the antigenic determinant in question. When this project was started, standard protocols, such as those present in manuals from biotechnology companies,

were used. The initial goal was to perform a few trials of IHC with a positive control Ab. A positive control, we reasoned, would allow us to convince ourselves that the methodology in use was adequate. But, while trying to perform our control IHCs, we encountered several technical challenges.

Upon tissue fixation, cross-linking bridges established by the fixative may prevent detection of antigenic sites. In order to circumvent this problem a mechanism that partially reverses or destroys such "artifacts" is needed. Such a process is commonly called "antigen retrieval". There are two major retrieval systems: heat-based or enzyme-based (as discussed in Supplement 2). Enzyme-based antigen retrieval often involves both the digestion of fixative cross-links and some means to increase tissue permeability, thus permitting Abs to more easily reach intracellular epitopes. Because Alk2 is a transmembrane-spanning receptor protein, such methodology seemed unfeasible and prone to lead to changes in expression patterns due to antigen degradation, loss, and/or redistribution. Therefore, we elected to use the heat-based protocol, however, our first attempt to process our tissue via heat-induced antigen retrieval (HIAR) showed the method to be remarkably deleterious. Complete tissue loss occurred when slides were either *a)* immersed in an antigen retrieval buffer and then heated by means of microwave irradiation or *b)* placed in a buffer-filled reservoir over a steaming platform. To minimize tissue loss from the slides we then decided to forgo the antigen retrieval steps, with the expectation that some

epitopes would escape masking during fixation and thus be available for binding to the Ab. The results of those experiments, however, led us to a different conclusion.

When anti-notochord Ab was directly applied over fixed and blocked sections that had not undergone antigen retrieval, no signal was observed, regardless of the Ab titer used. Ag retrieval seemed then unavoidable, and with it alterations to the protocol currently in use. As previously mentioned, the current immunohistochemistry protocol leads to loss of tissue when sections were submitted to antigen retrieval. It was only later, when commercially available charged slides were used instead of plain glass microscope slides (covered with Haupt's adhesive), that we were able to perform a successful Ag retrieval without complete loss of the tissue sections from the slide. Following this technical modification, we then observed that the control Ab would indeed highlight the notochord tissue as expected (Fig. 38).

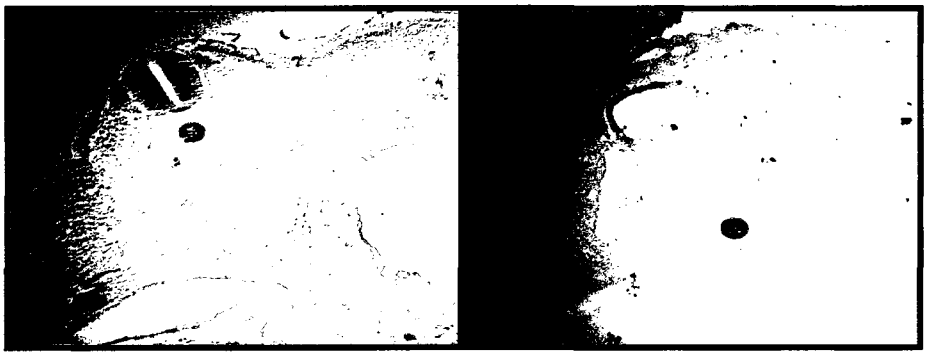

Fig. 38. Detection of notochord via IHC. 10μm section of embryo H1V1-3 (Harvest 1, vial 1, embryo 3). Sections were deparaffinized in xylene,

rehydrated and submitted to 20' steam based HIAR in citrate buffer pH6.0. Sections were incubated 1h20 min at 37 °C with 1:5 anti-notochord Ab and 1 hr (37 °C) with 1:2000 secondary Ab. Sections were counterstained with eosin and methyl green respectively. Harvest 1 embryos were embedded in paraffin using a slightly different protocol with generally longer infiltration times but results were reproducible with sections of embryos from harvest 6.

This protocol, however, is far from perfect. The use of charged slides prevented a complete loss of tissue during processing, but Ag retrieval was still able to cause random and extensive tissue damage. As a result, it was still impossible to obtain a series of sections of sufficient integrity to determine the expression pattern of any protein throughout the heart, let alone along the entire body axis. Additional attempts were made to optimize the protocol by assaying a variety of different conditions. Even our most successful protocol, however, still leaves much room for improvement. Tissue degradation and detachment during antigen retrieval remained a significant problem, even as the lab work for this thesis was being concluded and the manuscript being prepared. Degradation, in fact, still seems to be random from slide to slide and section to section, even in the absence of Ag retrieval. This variability may be due in part to *a)* "lot-to-lot" variation in the quality of the charged microscope slides we used; *b)* some subtle (and unknown) variation in our section swelling and mounting technique; *c)* the still relatively crude and difficult-to-reproduce method of steam-based antigen

retrieval we employ; *d)* some unknown instability in one or more of our reagents; or *e)* one of the countless other variables that might exist in this long and complex protocol. This thesis, however, describes a key effort to pioneer the use of more advanced histological techniques in our lab, to establish an experimental framework for optimizing our immunohistochemical protocol, and to provide preliminary data on the protein expression domain of Alk2 in the developing heart.

IMMUNOHISTOCHEMICAL OPTIMIZATION STUDIES:

Most IHC protocols that we found in the literature recommend a 4 hr fixation with 4% PFA, mounting of the sections onto common glass microscope slides, and a short deparafinization in xylene followed by heat-induced Ag retrieval. Extensive tests performed during this research, however, have demonstrated that every single step mentioned above should be carefully examined and optimized in order to obtain high quality, lasting sections. That said, here we present the most successful conditions for IHC in chicken embryos we have defined to date.

1 - Tissue harvesting and fixation

High quality tissue harvesting is crucial for the adequate preservation of the tissue in question. Often, embryonic tissues are surrounded by tightly

adherent protective membranes which can inhibit tissue penetration by most reagents. During harvest, such membranes should be removed as completely as possible (without damaging the extremely delicate embryo) to permit the penetration of dehydrating solutions and fixatives into the tissue. Upon successful harvesting the tissue must be immediately fixed. Routinely, 4% paraformaldehyde is used as a fixative in IHC and fixation is performed for 4 hr at room temperature (see supplement 1 for alternative fixatives). Alternatively, fixation can be done overnight at 4 °C. During this research, I examined the effects of cold overnight fixation *vs.* shorter fixation periods at room temperature. When embryos were harvested, rinsed in 1xDPBS, fixed at room temperature, embedded and processed for histological purposes, good quality, stably attached sections could be obtained. The tissue stained well and exhibited good tissue architecture. When similarly processed sections were submitted for IHC, the inclusion of the harsh heat-induced antigen retrieval step, produced severe degradation. In the trials performed, a 21 hr fixation period was found to be the most effective for maintaining tissue preservation when combined with the use of charged slides and heat-induced Ag retrieval. While the combination of a 21 hr fixation and the use of charged slides resulted in a superior tissue preservation, our problem was not completely solved and degradation was visible after IHC. Any number of variables, such as the quality of the charged slide coating, might cause or aggravate such a problem. One could also argue that environmental variables, such as water pH and temperature changes during the various stages

of tissue processing, might be influencing our results. We would expect, however, that such variables would affect equally all sections in one slide and all slides of one single test. However, this exasperating problem seems to be random, with degradation levels varying from slide to slide and section to section.

2 - Tissue dehydration and embedding

Upon fixation, tissue must generally be embedded in a solid media if tissue sections are required. Paraffin is a common embeding medium although, due to its immiscibility with aqueous reagents, it cannot be used directly upon fixation. Dehydration of the tissue with an alcohol gradient solution and alcohol back extraction with organic solvents is required before embedment. The trials performed at this step demonstrated that the election of reagents here is crucial for the success of the overall protocol. Although both ethanol and methanol are usually considered suitable for the dehydration protocol, and both are cited routinely in the IHC literature, the use of ethanol seemed to cause a higher degree of tissue shrinkage. When using ethanol it's also crucial to select high purity reagents. We found, for example, that the use of ethanol containing as little as 0.03% denaturants (often modified ketones) can lead to severe tissue shrinkage, reducing the tissue size by up to 80%. Such extensive shrinkage is likely to cause irreversible damage to the tissue and lead to a significant degree of structural artifacts upon sectioning.

Interestingly, when denatured ethanol was used in this dehydration procedure embryos maintained their original size until immersed in xylene[3]. At that time the xylene became temporarily opaque-white, possibly as a result of incomplete dehydration or presence of contaminants (used for alcohol denaturation) that could lead to light scattering. When retrieved from the xylene, embryos were, as stated above, remarkably reduced in size. This series of optimization experiments thus argues strongly for the use of either 200 proof ("Gold Shield") ethanol or pure methanol. The expense of 200 proof ethanol, however, is significant, and "methanol-denatured" ethanol might serve as a high quality substitute at a much reduced price.

3 - Sectioning technique and microscope slides

Tissue sectioning is likely the part of an immunohistochemistry protocol that depends mainly on the skills of each researcher. Good sectioning, which would result in the presence of even sections with no knife marks, is critical for tissue preservation during staining and high quality morphological observation of the tissues. While it is assumed that each researcher should learn and practice sectioning individually, a few useful tips, which can result in higher quality material, will be described in this section. The major point to consider when sectioning will be the type of procedures that will be used for staining the sections once they are cut. For regular histological analysis, a 10μm section laid

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3 Xylene is one of the most commonly used paraffin solvent (also known as "clearing" agent) for IHC, However, other organic solvents such as toluene, limonene, benzene and cedar oil can possibly be used as a replacement.

over a pre-warmed water-flooded glass slide (possibly coated with Haupt's adhesive), usually but not always, leads to great results. While this technique was usually sufficient for histological staining, a blocking step with PBS/BSA employed during the IHC protocol seemed to be sufficient to detach these sections from the slides. To my great surprise, when such slides were submitted to alcohol-based rehydration tissue sections were seen throughout all rehydration steps with no visible damage to the naked eye. However, adding the PBA over the tissue sections resulted in a fast degradation of the tissue. Visually, the tissue sections seemed to dissolve as the reagent was added. In an attempt to determine if BSA (or contaminating enzymatic activity with it) could be contributing to this tissue loss, acetylated BSA (acetylation destroys all enzyme activity) was used instead. That modification turned out, however, to produce but no better results. While no explanation was found for such a phenomenon it is possible that the nature of this BSA solution might interfere with the charges that bind the tissue to the glass slides, causing them to dislodge. In addition, the harsh procedures during antigen retrieval for IHC exacerbated this problem. The high temperatures achieved during antigen retrieval go well beyond the melting point for Haupt's adhesive (which is mainly composed of glycerin, for which the melting point is 18 ºC). The melt down of the adhesive at this high temperatures may further diminish adherence of the sections to the slides. Alternatively, we tried directly mounting sections onto slides in the absence of Haupt's adhesive. We found that mounting sections over raw glass does not allow a strong enough

adhesion when submitted to antigen retrieval in a solution where convection currents will occur. This way, tissue must be mounted onto charged slides (such as Fisher brand Excel Superfrost slides, as recommended by Loretta Chan, UCSF). However, while charged slides might be a requirement for good adhesion, they alone are not a solution. When sections are laid over a glass slide only the first layer of cells is able to interact with the glass and the charges in the coating solution in use. Using charged slides will thus not benefit any other successive layer of cells. Thus, adequate fixation of the tissue must be performed, so that the different layers of cells are bound together to the glass via the intermediate cell-to-cell bonds established by the fixative.

Unexpectedly, the use of charged slides poses some novel challenges. The conventional methods for mounting a section of ribbon on a slide must be modified or damage to the sections can ensue. When charged slides are locally flooded with water the charges present at the surface of the glass prevent the water from spreading easily, forming a "water droplet" on the slide. When sections are then applied over this water droplet, the paraffin section moves around until it binds to the glass surface, giving neither the time for the paraffin to expand nor for the researcher to correctly position the ribbon of sections. When the water is then absorbed via capillarity using a Kimwipe, the paraffin sections are attracted to the glass even before all the water is removed. This effect causes one or more small water droplets to be enclosed under the paraffin

ribbon. To circumvent such problems the paraffin sections can alternatively be laid down over a 40 °C water bath. Note that the water bath reservoir shall be previously scrapped with a spatula or similar implement to remove any adherent air bubbles at the bottom or sides of the reservoir. When the paraffin section expands a charged slide can be immersed in the water in a perpendicular position and brought in contact with the floating ribbon (Fig. 39). Upon allowing the first section to adhere to the glass the slide must be slowly pulled out of the water in a perpendicular plane (60-80° relative to the water surface).

Fig. 39. Mount of paraffin-embedded tissue onto charged microscope slides. A ribbon of paraffin sections is laid over a temperature controlled 40 °C water bath. Upon expansion of paraffin, a charged microscope slide is immersed in the water and, with the slide at a 45-60° angle, brought in contact with the ribbon. Upon successful adhesion of the first section to the glass, the slide is pulled out of the water, slowly at a 60-80° angle. If such method is used with non charged slides extra attention must be

given as the ribbon might slide away when removing the slide from the water.

This method will allow the water present between the slide and the ribbon to run down off the slide as the ribbon and slide come together. Slides should then be air dried in a perpendicular plane for no less than 1 hr (2 hr gives good results). When all obvious residual water has been removed, the slides should then be dried at 37 °C to ensure that no humidity remains between slide and paraffin sections. The positioning of the slides during this period is also crucial. If slides are placed horizontally and warmed to 37 °C in a hot plate (as many conventional protocols require) it is possible that residual humidity beneath the paraffin ribbon can burst through it as it warms up. Water evaporation will sometimes cause massive tissue destruction. If instead one uses an oven, set for 37 °C, it is possible to position the slides in a non-horizontal angle relative to the surface (Fig. 40). Keeping the slides at an angle will permit the evaporating water to be released without bursting through the paraffin. The following picture (Fig. 41) depicts 2 slides. Panel A shows a slide dried in a horizontal position on a hot plate set for 37 °C. Panel B shows a slide dried in a vertical position in an oven at 37 °C.

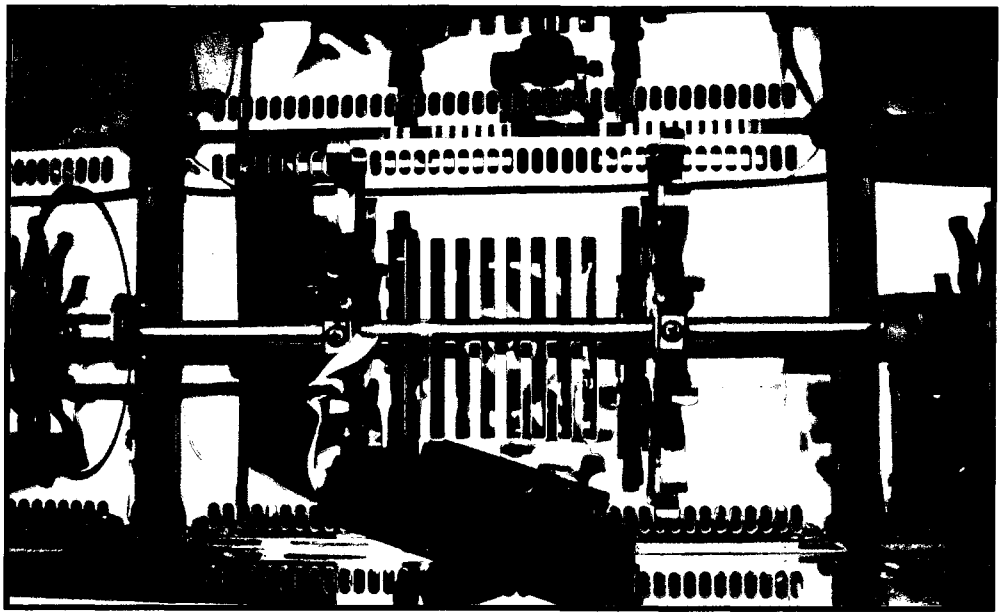

Fig. 40. Baking of sectioned tissue. Recently (1-2 hr) sectioned tissue is baked at 37 °C at a slight angle to permit better adhesion without tissue disruption by the evaporating residual water. The oven depicted in this picture is an hybridization oven but any other oven capable of keeping the temperature at 37 °C can be used.

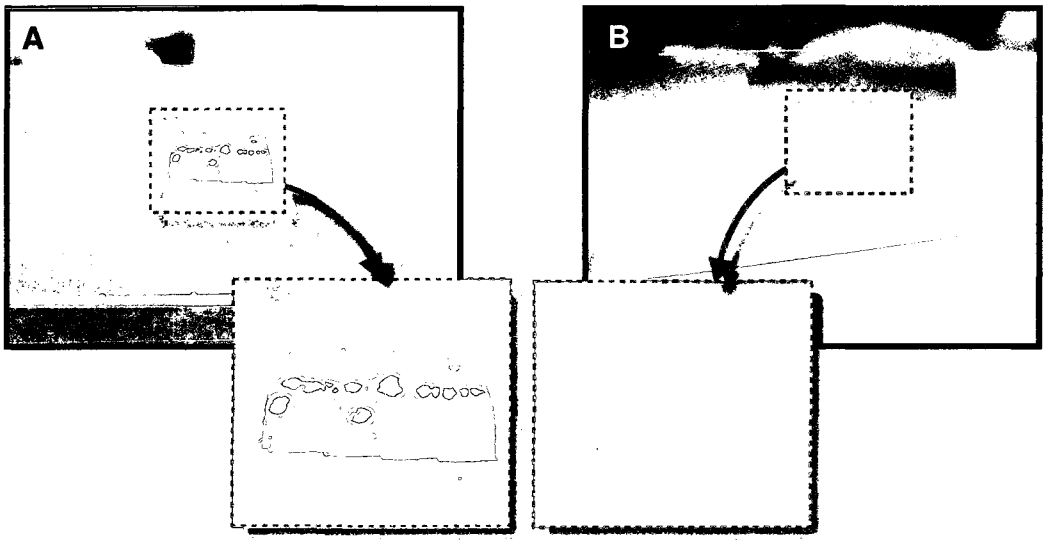

Fig. 41. Section baking horizontally over an hot plate vs. vertically in an oven. Panel A shows the results of a paraffin ribbon baked over non charged slides on a 37 °C hot plate. Slide was dried in a horizontal position leading to water evaporation and burst through the paraffin. On panel B is depicted a charged slides which was baked on a 37 °C oven. Slides were kept at an angle as demonstrated on Fig. 40. Drying slides in a non horizontal position permitted the evaporating water to escape between the section and slide preventing bursting of the tissue.

As an alternative to the use of charged slides (which can be fairly expensive), coating glass slides with "Mayer's albumen glycerol" represents an easily done option. This solution can be prepared by mixing equal parts of egg white and glycerol followed by filtration at 55-58 °C (Lillie, 1948). Albumin coating has been recommended by Karen Jensen at the Developmental Studies Hybridoma Bank, Iowa University.

4 - <u>De-waxing and section dehydration</u>

So that the mounted tissue can be treated for immunohistochemical staining, paraffin must be removed so that Abs can have free access to their antigenic epitopes. "Dewaxing" is commonly done by briefly immersing the slides in xylene. However, as recommended by Loretta Chan at UCSF, a brief de-waxing step might not be sufficient to completely remove all the surrounding paraffin. If paraffin remains in the tissue, the paraffin will melt during the antigen retrieval step weakening the adhesion of the sections to the glass (note that Ag retrieval buffer during Ag retrieval reaches around 95-98 °C, well above paraffin's melting point). In order to improve tissue adherence it is recommended that slides be initially de-waxed by simply melting the paraffin in a 60 °C oven. During this step the position of the slides is important to prevent loss of tissue sections. It is recommended that slides are kept at the same perpendicular position as before (If the slides are placed in the horizontal position the paraffin would cause the sections to lift and float. Putting the slide in a vertical position might lead to loss of the tissue). This heat-induced de-waxing allows the paraffin to melt away from tissue. As the paraffin melts away it is possible that more cells, previously coated with the paraffin, will contact with the glass permitting a stronger adhesion. Paraffin melting should not extend past 1 hr because such a prolonged exposure of de-waxed tissue to high heat might lead to loss of antigenicity (other researchers, including the technical support for the

Superfrosted Excell slides in use, commonly perform a 15 min heat-based de-waxing). Upon heat-based dewaxing, the slide should be cleared with xylene for a total of 3 changes of 5 min incubation for each change. This demonstrated high quality results without compromising the signal when using our anti-notochord positive control.

Slides will then be ready for dehydration. Dehydration is a pretty straight forward procedure with not much to error. Slides are briefly (3-5 min) and sequentially immersed in a down gradient of alcohol-water. Although, one recommendation should be taken into account. When "finicky" tissue is in use, immersion of slides in each solution should be done slowly. Specially when transferring slides between different solutions such as xylene based to ethanol based solutions the brownian movements created during solution miscibility are sometimes enough to disrupt tissue adherence. The use of ethanol is know to have a double role. It not only dehydrates the tissue but aids on tissue fixation via protein coagulation.

Following dehydration slides are usually submitted to Ag retrieval. During this research a second backing step at 37 ºC was considered upon dehydration as a possible solution to increase tissue to glass adhesion. However, while the first trial seemed a remarkable success, such baking step did not seem to provide consistent results. Other scientists perform a second fixation over the deparaffinized and rehydrated sections. The higher penetration ratio of

methylene glycol than formaldehyde (see supplement 1) and the low speed conversion of methylene glycol to formaldehyde (which is based on temperature) leads to usually under fixed tissue cores, with adequate fixation of the surroundings. Re-fixing the deparaffinized tissue might lead to a more even immunostaining and lower tissue degradation.

5 - Antigen retrieval

In this research the only antigen retrieval system tested was a heat-induced retrieval in citrate buffer pH6.0. While the use of steam, microwave or pressure cooker might lead to different results, steaming was efficient in this case. Antigen retrieval under steaming conditions has the advantage of permitting a more controlled and even temperature distribution and thus was the method elected. In my experience, placing a large beaker, containing one inch of water, over a controllable heating plate works great as a steaming platform. Away from the water (1 to 2 cm), conical 50ml centrifuge tubes must be placed horizontally and filled with citrate buffer. The heat is turned on and slides are immersed in the buffer as soon as it reaches 40 °C. Tubes are loosely capped, and the beaker covered with a loose petri dish. Upon 20 min incubation, tubes must be removed and allowed to cool down. Attempting to use the slides immediately would suddenly dry the tissue causing possible damage. Concomitantly, the sudden change in temperature could cause the tissue to detach from the glass.

Ag retrieval is still a very controversial technique whose underlying theory is not understood. However it is important to remember that different epitopes/ Abs might required different levels of Ag retrieval. A 20 min retrieval was suitable and the only form to obtain signal when using an anti-notochord Ab, however, it often lead to high background noise when applied together with anti-Alk2 Abs. The reason might be both the nature of the residues that constitute each epitope and/or the linear or tridimensional rearrangement of the epitope in elected Ags. While notochord epitopes might be preferentially fixed due to a possible richness of commonly cross-linkable residues, Alk2 epitopes might be more randomly accessible to the respective Abs due to less fixation based masking. On the other hand, most Abs are designed to interact with linear peptides but many linear peptides within a protein are masked by the protein internal folding. The high temperatures achieved during Ag retrieval might not only permit destruction of fixative cross-links but also linearize many peptide epitopes via denaturation. Epitope linearization could not only explain the less understood side of Ag retrieval, where no clear explanation exists for how the different methodologies influence Shiff and methylene bridges, but also would explain the increase of background noise in the presence of certain Abs. Local denaturation of fixed proteins (maybe together with disruption of Shiff bases) might improve exposure of those linear peptides within a tridimensional protein for the Abs in use. At the same time, highly related epitopes will be unmasked leading to an increase in background noise. The presence of highly related epitopes might also be

explained based on the nature of the peptide used to raise such Abs. According to the product data-sheets the anti-notochord Ab was raised against a notochord protein extract. The Abs thus produced are likely targeting tridimensional epitopes on native proteins. Such Abs are often very specific. The sequences of amino acids of the epitope and its tridimensional conformation makes it unlikely that a different protein would share a common configuration decreasing the likelihood of Ab cross reactivity. This would permit cleaner results even upon Ag retrieval. Ag retrieval would only permit a better exposure to very specific epitopes by breaking cross-linking bonds that would otherwise slightly change the distance between amino acids in a tridimensional structure. On the other hand, SCBT anti-Alk2 was raised against an antigenic peptide. Antigenic peptides in solution are often linear and flexible. As Abs are raised, such peptides can achieve different conformational stages. Using such a flexible epitope would result in a combination of Abs that can bind to many distinct tridimensional epitopes. Randomly created non physiological tridimensional epitopes increases the likelihood that one or more of this epitopes actually closely resembles epitopes in proteins different from Alk2. When an anti-Alk2 Ab is presented to a fixed tissue it will react only with the epitopes with which it has higher affinity. In the presence of Ag retrieval, the unmasking of an high concentration of epitopes of low affinity might interfere with the Ab binding specificity.

The duration of Ag retrieval is crucial to achieve a stage where enough unmasking/denaturation occurs to improve specific signals without increasing unspecific binding. This might explain why no results where obtained with anti-notochord Ab without Ag retrieval being the opposite for anti-Alk2 Abs. A positive control in IHC is something not easy to provide and not always useful. As we demonstrated, the Ab destined to work as positive control and the Ab for our reaction, work under different conditions making little sense of the word "positive control."

For more information regarding fixatives and antigen retrieval techniques please see supplements 1 and 2.

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SUPPLEMENT 1 - FIXATION AND FIXATIVES

Fixation Purpose

Fixation is a common technique that precedes any histological and cytological protocol and which major function is the preservation of tissue or cells from degradation: intrinsic (by autolysis) or bacterial/fungal (Woods and Ellis, 1994; Key, 2006). Fixation can also protect cells from the harsh procedures commonly employed during histology and immunochemistry (note that simple steps such as a water rinse can irreversible affect the sub-cellular structures due to osmotic movements) at the controversial cost of chemical and physical alteration of molecular properties (such as protein denaturation, epitope masking, breaking down of membranes with loss of small molecules) (Key, 2006).

Alternatively, preservation can be achieved by freezing the tissue of interest. Freezing based protocols have the advantageous characteristic of preserving epitope antigenicity since no masking chemicals or denaturing temperatures are needed, and allowing tissue to be sectioned with no need for embedment while the low temperature prevents autolysis. Although it is known that resolution is often inferior than in paraffin-embedded tissue sections after chemical fixation. Sometimes, for optimal results, fixed tissues is frozen and sectioned.

Types of fixatives

A wide list of fixatives, with different properties and fixation mechanisms, exist (Key, 2006). Because of such a diversity, no fixative is adequate to the fixation of all antigens. Selection of the appropriate fixative or a combination of two thus need to be employed (Woods and Ellis, 1994). While there is no fixed rule for selecting one or another fixative and commonly personal experience dictates, understanding how fixation can be achieved might help during reagent selection.

Classification of fixatives is often ambiguous but usually arranged in two major classification groups: method of fixation (coagulant Vs non-coagulant) and category of fixative (alcoholic, aldehydic and heavy metal fixatives) (Woods and Ellis, 1994).

As presented by Woods and Ellis, (1994); fixatives can be classified as follows:

- Aldehydes and other cross-linking fixatives (formaldehyde, glutaraldehyde)
- Oxidising agents: metallic ions and complexes, such as osmium tetroxide, chromic acid.
- Protein-denaturing agents, such as acetic acid, methyl alcohol (methanol), ethyl alcohol (ethanol).

- Unknown mechanism, such as mercuric chloride, picric acid.
- Combined reagents.
- Microwaves.
- Miscellaneous: excluded volume fixation, vapor fixation.

[for more information on this matter refer to Hopwood D. Fixation and Fixatives. In: Bancroft JD & Gamble M. Theory and Practice of Histological Techniques. 5th edition, Edinburgh: Churchill Livingstone. 2002]

Aldehydes and other cross-linking fixatives: Aldehyde fixatives are thought to create cross-links between proteins into a mesh network that holds soluble proteins to structural ones. This way proteins are kept in their original location and *in vivo* molecular relationships are preserved (Woods and Ellis, 1994). The following list of fixatives shows the most commonly used aldehydes for histology and immunochemistry. Many other exist and can be found in the literature.

Formaldehyde: Formaldehyde is a gaseous compound at room temperature with the molecular formula H_2CO. It is a natural compound present in smoke and results of the oxidation of methane and other carbon compounds. Due to its high solubility in water, it is sold commercially in

aqueous solutions generally called formalin/formol. Formalin solutions has a saturation point of 40% formaldehyde in water (Mason, 1991; Lillie, 1948). After reacting with water (Fig. 42) formaldehyde becomes methanediol (methylene glycol) with the molecular formula $CH_2(OH)_2$ (Fox, 1985; Mason, 1991; Hayat, 2002). According to Hayat (2002), is the presence of methylene glycol that turns a formaldehyde solution a fast penetrant and the conversion between methylene glycol and formaldehyde is temperature dependent, with higher temperature favoring formaldehyde production.

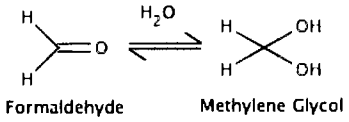

Fig. 42. Conversion of formaldehyde and methylene glycol.

Because of the tendency of formaldehyde to reversibly polymerize to cyclic trimer trioxane or paraformaldehyde, formalin is actually usually available as a mixture of 37% formaldehyde, 10-15% methanol (to limit the extension of polymerization) and water (neutral salts and a buffering system is commonly added to control pH and maintain tonicity of the tissues - ex. PBS). Note the ambiguity of describing formaldehyde concentration where some commercial manufactures label 40g formaldehyde in 100 ml of water and other 40g in 100g of water (causing a

40% m/w to become 37% m/m) (Fox, 1985). The use of methanol became a common preservative since, chemically, formaldehyde is produced from the "catalytic oxidation of methanol (Fox, 1985) and commonly stored as anhydrous paraformaldehyde polymer. "Formaldehyde has a natural tendency" to be readily oxidized by atmospheric oxygen and form formic acid (Fox, 1985; Lillie, 1948). Small amounts of formic acid is found in commercial formaldehyde and can produce unwanted pigment precipitation in blood-rich fixed tissue (Fox, 1985). Because formic acid reduces formaldehyde preserving capabilities neutralization of the solution is a requirement (Woods and Ellis, 1994). Alternatively, addition of calcium carbonate, which reacts with formic acid producing a neutral solution, or ion-exchange chromatography to reduce hydrogen and formate ions, can be employed (Fox, 1985).

It is believed that formaldehyde causes tissue fixation by cross-linking primary basic amino groups in proteins (such as lysine) in the outer side of a folded protein (Woods and Ellis, 1994) via the formation of Schiff[4] bases (Mason, 1991) with other nearby nitrogen atoms. As presented by Woods and Ellis (1994) it is also believed that formaldehyde can react with imino and amido groups (to form hydroxymethyl compounds which react with further amide moieties to form methylene diamides, as mentioned in

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4 Schiff base: Imines bearing a hydrocarbyl group on the nitrogen atom R2C=NR' (R' ≠ H). Considered by many to be synonymous with azomethines (McNaught, 1997).

Mason (1991); also called methylene bridges (Key, 2006; Hayat, 2002)). Woods, Ellis (1994) and Mason (1991) also mention the possibility of interaction with guanidyl and alcoholic hydroxyl groups to form acetals, carboxyl and sulfhydro groups to form sulfhydral acetal analogues and aromatic rings or hydroxymethyl groupS in the formation of cross-linking bonds. According to Sompuram "in solution, formaldehyde is capable of binding to the following amino acids: lysine (K), arginine (R), tyrosine (Y), asparagine (N), histidine (H), glutamine (Q), and serine (S)" (Sompuram, 2004; Hayat, 2002). Rections with other amino acids such as tryptophan, cysteine, are also discussed by Hayat (2002). By understanding protein folding and amino acid composition of the epitope to be detected one could predict how much of that epitope will be masked by cross-linking. As described by Key (2006), "if formaldehyde reacts with amino acids within the epitope, the antibody will be unable to bind and therefore will be of no use in formaldehyde-fixed tissue". Using a different fixative or decreasing fixation time and the use of polyclonal Abs might be recommended.

In Immunohistochemistry procedures, 4% paraformaldehyde in water (or 10% formalin) is commonly used (Woods and Ellis, 1994; Key, 2006; Lillie,1948). Due to its small molecular weight formaldehyde is a fast penetrative agent but known to be somewhat slow fixative (Fox, 1985;

Hayat, 2000). The reason being the need of conversion of methylene glycol (hydrated formaldehyde) into formaldehyde for the establishment of bonds with nearby proteins (Fox, 1985; Hayat, 2000). Fixation is commonly done for 4 hr at room temperature or overnight at 4 °C (since it causes no deleterious effects on the cell morphology) (Woods and Ellis, 1994; Key, 2006). During tissue fixation with formaldehyde not all tissue is uniformly fixed. The inner areas of a tissue block are often just exposed to the fast penetrant methylene glycol, and thus fixed via coagulation during dehydration in ethanol (Hayat, 2002). While cold fixation prevents autolysis, room temperature speeds up the process (due to higher conversion of methylene glycol to formaldehyde) but it is not recommended during overnight fixation (Lillie, 1948). Time and temperature should be controlled in a way to prevent over-fixation which can be a problem if epitope masking is achieved. In a study made by Sompuram (2006), complete fixation of tissue-free epitope is achieved in 16 hr. While fixation will take longer when whole tissue is employed, an increase of temperature will lead to an approximation of times and cause over-fixation (Sompuram, 2004). While over fixation can cause epitope masking tissue hardness is maintained constant, making of formaldehyde a very versatile fixative for histology (note that by leaving all tissue at the same hardness point facilitates equal sectioning in paraffin) (Fox, 1985).

4% PFA is usually added of 2% phenol for an "improved nuclear and cytoplasmic detail, reduced shrinkage and distortion" (Woods and Ellis, 1994). As mentioned by Woods and Ellis (1994), "a concentrated solution of formalin sometimes becomes turbid on storage through the production of paraformaldehyde which decreases the strength of the solution, but can still be used as a fixative following filtration. Formalin favors the staining of acidic structures (nuclei) with basic dyes and diminishes the effect of acid dyes on basic structures (cytoplasm)".

While formaldehyde is commonly the fixative of election for light microscopy immunohistochemistry (due to a lower epitope masking) it is not recommended for electron microscopy. The levels of tissue preservation it permits do not allow for good morphological detail under EM high magnification.

Glutaraldehyde: Glutaraldehyde has the molecular formula $C_5H_8O_2$ and it is another commonly used fixative (more so in electron microscopy). Due to it's bigger molecular structure is commonly used for small blocks of tissue (since big blocks might not be fully penetrated) and usually as a mix with formaldehyde (Woods and Ellis, 1994). One of the reasons being that glutaraldehyde is more prone to create irreversible cross-links that are not recovered by known antigen retrieval techniques. This way, glutaraldehyde leads to an higher epitopes masking and might

be less suitable for IHC, but does permit higher tissue preservation crucial for improved details under electron microscopy (Hayat, 2002). Its mixture with formaldehyde (a faster penetrant and lower fixative) permit a good preservation in a fashionable time frame with out complete loss of antigenic reactivity. Glutaraldehyde can exist as aqueous solution with a complex formulation of 4% free aldehyde, 16% monohydrate, 9% dihydrate and 70% hemiacetal (Woods and Ellis, 1994).

Acrolein (acrylic aldehyde): Acrolein (also called propenal) is the simplest unsaturated aldehyde (with the molecular formula C_3H_4O) and can be produced by decomposing glycerol at high temperatures or oxidation of propane (Zang, 2008; Watanabe, 2007). It is a very strong fixative which produces more x-links than any of the other fixatives but has the disadvantage of low stability and polymerization by light. It is advantageous for enzymatic analysis since it can preserve the most labile enzymes. It is commonly employed as 4% acrolein (Woods and Ellis, 1994).

Others: As mentioned by Woods and Ellis (1994), "Glyoxal (ethanedial, diformyl), malonaldehyde (malonic dialdehyde), diacetyl (2,3-butanedione) and the polyaldehydes are other aldehydes which have been infrequently employed for fixation, mostly for special situations, to retain specific enzymes or proteins for histochemistry."

Oxidizing agents (metallic ions and complexes: osmium tetroxide and chromic acid):

Osmium Tetroxide: Osmium Tetroxide is a volatile solid of molecular formula OsO_4. Osmium Tetroxide is colorless while pure but the presence of osmium dioxide (OsO_2) gives it a yellow color. It is soluble in carbon tetrachloride and partially soluble in water in which becomes osmium acid. It is both a fixative and a lipidic staining used in optical and electron microscopy (Thompson, 2004). As a fixative is probably the most commonly used metal ion fixative but its low penetration prevents its use in light microscopy (causing tissue to crumble when embedded in paraffin) (Woods and Ellis, 1994). It is believed that Osmium Tetroxide reacts via protein cross-linking (as seen by the increased protein viscosity after OsO_4 treatment) but it is believed that fixation is mainly due to interaction with unsaturated lipids causing their stabilization via "oxidation of double bonds between adjacent carbon atoms to form monoesters and diesters, the binding of lipid to protein and the conversion of unsaturated fatty acids to stable glycol osmates" (Woods and Ellis, 1994). Osmium tetroxide can interfere with histological stains (Woods and Ellis, 1994; Lillie, 1948). It is usually employed as a secondary fixative after glutaraldehyde for electro-microscopy. While this is advantageous for electron microscopy and

causes good membrane preservation it is not adequate for light microscopy (by reasons mentioned above) (Key, 2006).

Chromic Acid: Chromic acid is a compound usually mixed with many other reagents and acts as a strong oxidizer. Due to its low permeability and tendency to predispose tissue for shrinkage it is not commonly used for fixation (Woods and Ellis, 1994).

Protein-denaturing agents:

Methanol/ Ethanol: Methanol (CH_3OH), the simplest alcohol, and Ethanol (CH_3CH_2OH) are frequently used as fixatives. Both alcohols are believed to disrupt protein tertiary structure (via destruction of hydrophobic bonds) while preserving secondary structures (since hydrogen bonds are more stable in alcohol than water) (Woods and Ellis, 1994). Due to it's smaller molecules methanol is slightly more permeable, and according to Woods and Ellis, due to it's close relationship to water molecules it strongly competes with water for hydrogens bonds causing not only fixation but tissue dehydration, the same occurs with ethanol but with lower intensity. Cytoplasmatic shrinkage, histone denaturation with consequence nuclear distortion, DNA/RNA extraction is possible if fixation is prolonged. Although glycogen, pigments and blood is well preserved by

absolute ethanol (Lillie, 1948; Woods and Ellis, 1994). No other alcohol has fixative properties (Woods and Ellis, 1994).

Acetic Acid: Usually added to ethanol/methanol for faster fixation preventing extensive shrinkage (Woods and Ellis, 1994). Possibly mixed with Zinc Chloride which allow good preservation of membrane proteins. The latest can be formulated as follow: Zinc chloride 500 g, Formalin (40% w/v formaldehyde) 3 L, Glacial acetic acid 19 mL, Distilled water 20 L (Key, 2006).

Unknown mechanism, such as mercuric chloride, picric acid:

Picric Acid: "When used in combination with other ingredients, leaves tissue soft and penetrates well, precipitating all proteins" (Woods and Ellis, 1994). However, when applied alone usually leads to quit hard fixation (Lillie, 1948). "It will continue to react with the tissue structures and cause a loss of basophilia unless the specimen is thoroughly washed following fixation" (Woods and Ellis, 1994). Picric acid is highly explosive and thus saturated picric acid (1.17 g/100 mL distilled water) should be used (Key, 2006).

Bouin's solution: This solutions is the result of mixture of cross-linking fixatives with fixatives of unknown mechanism (saturated (1.2% w/

v) aqueous picric acid 75 ml, formalin (40% w/v formaldehyde) 25 mL, glacial acetic acid 5 ml) (Key, 2006; Lillie, 1948). It is a fast and strong fixative, however different sources recommend varied different fixation time (from 1-2 hr until several days) (Lillie, 1948; Key, 2006), with low fixation capacity of kidney tissue. Prolonged fixation causes brittleness. Precipitates can form if not properly washed. Lipids are destroyed or reduced (Key, 2006).

Mercuric Chloride: Mercuric Chloride based fixatives are fast penetrating fixatives that precipitates most proteins by interacting with a wide variety of amino acids (Woods and Ellis, 1994). They are usually prepared in mixture with other fixatives or used prior an initial fixation in formalin for a more complete fixation. They exist in association with neutral salts to preserve tissue tonicity (Key, 2006). In opposition to what is suggested by Woods and Ellis (1994), Key (2006) describes such fixatives as "poor penetrators" and thus small blocks of tissue should be employed. Are believed to be coagulative but their form of action was not yet described (Key, 2006). They were commonly used in the past (Key, 2006; Woods and Ellis, 1994) prior to the time when tissue was embedded in paraffin (Key, 2006). The coagulative properties of Mercuric Chloride hardens the tissue to the point of possible sectioning. Maybe due to its high toxicity and crystal deposition in the tissues (implying extensive tissue

washing) are rarely used nowadays (Woods and Ellis, 1994). They are recommended for cytoplasmic antigens since they increase cell permeability allowing easy penetration of ABs. Like aldehydes they are additives and thus will cause epitope masking if exposure is prolonged and thus might not be suited for the use with monoclonal ABs. They have great cytological preservation (Key, 2006) and exists as Zenker's, Helly's, Ridley's and B5 solutions (Woods and Ellis, 1994; Key, 2006).

Acetone: Acetone is a highly volatile liquid that works as a fast fixative but cause tissue brittleness if exposer is prolonged (Woods and Ellis, 1994). It is considered one of the best histological preservatives (even more than cryofixatton) since it does not destroy immunoreactive sites (Key, 2006; Woods and Ellis, 1994). In respect to the penetration speed of this reagent some controversial ideas exist. However, to improve penetration it is usually employed as a mixture of acetone-methylbenzoate-xylene and must be cleared with "methylbenzoate and xylene before paraffin embedding" (Woods and Ellis, 1994) or as complement for cryofixation or smears (Key, 2006). It is a great fixative (relative to alcohol of formalin) for fixation of phosphatases and lipases (Lillie, 1948)

Combined reagents:

Microwaves: The appearance of highly controllable heating mechanisms such as microwaves have allowed the use of heat as histological fixative. It seems like temperatures between 70 and 85 °C are able to preserve tissues in a tissue dependent fashion. If temperature is not tightly controlled tissue destruction occurs rendering old heating methods unfashionable. Microwaves are perfectly suited for this application because due to their 2.5GHz non-ionizing radiation emission, particles containing dipolar molecules (water, protein side chains...), are constantly and instantaneously oscillated through 180° at the rate of 2.5 billion cycles per second. Tissue size is a limitation. Not only the heat created but also the acceleration of particles with increased collision might contribute for acceleration of chemical reactions. Microwave irradiation can be used in conjunction with other fixatives to decrease solution viscosity and thus increasing fixative penetration. Fixation by heat must be done in saline (unless fixatives are used) and can be as fast as 2 min. Antigenicity preservation is thought to be higher than in formalin fixed tissue but cannot be used with heat sensitive enzymes (unless unaltered substrates want to be assayed). (Woods and Ellis, 1994; Bancroft, 2002)

Miscellaneous: excluded volume fixation, vapor fixation.

SUPPLEMENT 2 - ANTIGEN RETRIEVAL

Antigen Retrieval

Antigen Retrieval is a controversial subject employed by most immunohistochemists to improve Ab binding. While the need is clear (fixatives might mask antigenic epitopes) the fundaments underlying it are not.

Epitope masking during fixation and heat caused denaturation (which can occur during paraffin embedment) are responsible for epitope destruction and thus low immunohistological staining (Key, 2006). While heat denatured epitopes are believed to be irreversibly changed (and the only solution possible is the change of embedding/hardening techniques) chemical fixation is usually partially reversible.

It is believed that formaldehyde causes tissue fixation by cross-linking primary basic amino groups in proteins (such as lysine (K) arginine (R), tyrosine (Y), asparagine (N), histidine (H), glutamine (Q), and serine (S)) in the outer side of a folded protein (Woods and Ellis, 1994; Sompuram, 2004) with other nearby nitrogen atoms causing alteration of the conformational shape of the protein being fixed (Hayat, 2002; Key, 2006). Because epitopes are mainly conformational (with a few being linear) the presence of methylene bridges will mask epitopes directly or by binding epitope-neighboring amino acids causing epitope-conformational changes. When epitopes are affected directly it is likely that no chemical treatment will recover its antigenicity. Although when only

nearby amino acids are affected, destroying cross-linking bridges might recover epitope conformational shape (Key, 2006). Sometimes, Ag retrieval can also lead to partial protein denaturation which will expose linear peptides previously masked within the internal protein folding.

A wide variety of mechanisms and reagents were identified as antigen retrieval agents. Temperature, pH and buffer composition and molarity appears to influence greatly antigenicity upon fixation (Sompuram, 2006; Hayat, 2002). Several theories exist to explain how antigen retrieval can influence reactivity. While some imply that methylene bridges cross-linking proteins after formaldehyde fixation are broken-down during retrieval, others believe that antigen retrieval is purely based on the remotion of residual amounts of paraffin, diffusion of blocking non-fixed proteins and rehydration of tissues (Sompuram, 2006). Evidences exist demonstrating that tissue subjected to Ag retrieval is not only faster penetrated by Abs but also that protein conformation changes. This evidences might be correlated with the destruction of cross-linking bridges that in a well fixed tissue leads to the creation of a tight protein network that sieves floating Abs (Hayat, 2002).

Different epitopes might have different responses to formalin fixation and Ag retrieval. Sompuram described 3 classes of antigens: those that loose reactivity after fixation but can recover after Ag retrieval techniques, those that are not affected by any of the treatments and those that are affected by formalin

only in the presence of other proteins but can still be recovered. This properties are apparently related with the amino acid content of each epitope but no concrete explanation exists yet. It is also important to note that some level of fixation is always achieved by coagulation during tissue dehydration with ethanol. Such fixation, which is usually the result of protein hydrolysis cannot be recovered by any Ag retrieval system (Hayat, 2002).

Types of Antigen Retrieval

One way around epitope masking is the use of polyclonal ABs. Although, and specially when the protein of interest is of low abundance one of the treatments described bellow should employed. It is of important notice that just like during fixation no single retrieval mechanism is known to produce optimal results for the antigen in study.

Proteolytic Induced Epitope Retrieval (PIER): Practical knowledge has shown that the use of enzymes such as trypsin, pepsin and pronase can, when incubated with the appropriate buffer, increase antigenicity. Most likely antigen retrieval is the result of digestion of cross-linking bridges and membrane degradation permitting Ab permeability. While no fixed digestion time is known, digestion should be proportional to the conditions employed during fixation (longer times for longer and hot fixations) (Millipore). The use of enzyme should be tightly controlled since false antigenic sites might be created (Millipore) and

membrane antigens might be lost. According to Key (2006), bromelan, chymotrypsin, and ficnin also have retrieval properties.

Heat Induced Epitope Retrieval (HIER): Temperature based antigen retrieval can be achieved in multiple several ways:

- Steamer heating
- Pressure cocker heating
- Autoclave heating
- Microwave oven irradiation

It is believed that all this techniques are based on the same principals where heat helps removing any residual paraffin and destroying cross-linking bridges. While the ideal temperature was never demonstrated, near boiling water temperature is commonly used (Key, 2006) and the duration of heating is of about 20 min (Key, 2006) (but can vary between 10 to 60 min). The amount on antigenicity recovered is proportional to the time and heat (Key, 2006; Hayat, 2002) and should be related with the duration of fixation (Hayat, 2002). Antigen retrieval is usually conducted in an aqueous buffer and "most retrieval solutions share a pH near two, seven or 10" (Key, 2006). Citrate buffer ph6.0, TRIS-EDTA pH9.0 or simply EDTA pH8.0 are commonly used. Although, 0.01 M TRIS-HCl, pH1 or 10 was proven to be slightly superior to citrate buffer of pH6.0 (Key, 2006). Buffers are usually detergent based to facilitate Ab penetration. However, in my personal opinion, membrane epitopes can be destroyed by this method. The use of EDTA as an Ag retrieval solution is based on the chelation of

endogenous calcium ions that can bind epitopes and mask them by hiding or changing their conformational shape (Hayat, 2002). Following retrieval, slow cooling of the retrieval solutions appears to be important (Key, 2006).

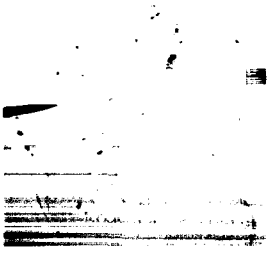
As mentioned by Key (2006) "several studies have now confirmed that pressurized systems supporting higher temperatures (up to about 120 °C) have yielded superior results in terms of intensity and number of sites stained, compared to non-pressurized systems operating at lower temperatures". Although, according to Millipore, pressure cooked sections usually present lower cytological detail. Hayat (2002) also defend that Ag retrieval can be improved by microwaving slides in buffer by a mechanism not much different from those used to defend microwave based tissue fixation.

It is believed that heat is able to recover antigenicity before denaturing proteins due to the fact that formalin based cross-linking can exist as methylene bridges or Schiff bases. While Schiff bases can be destroyed by temperature causing recovery of some antigen conformational shape, methylene bridges are not destroyed by temperature and hold proteins structure (partially) together (Key, 2006).

[For a more information regarding antigen retrieval and related protocols, the reader is directed to Kumar et al., 2008.]

SUPPLEMENT 3 - H6-4 EMBRYO SERIAL SECTIONING IMMUNOSTAINING

Canon PowerShot SD950 IS, 1/320, f/9,0, 9,57mm, ISO 200



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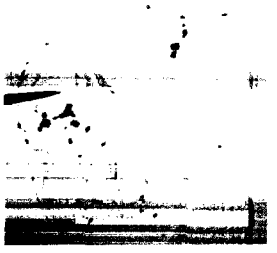
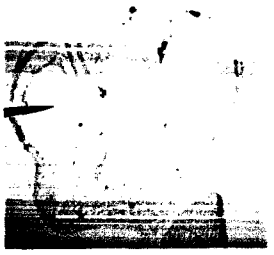


Canon PowerShot SD950 IS, 1/400, f/9,0, 9,57mm, ISO 200



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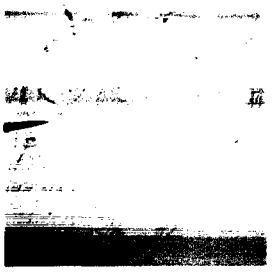
Canon PowerShot SD950 IS, 1/320, f/9,0, 9,57mm, ISO 200



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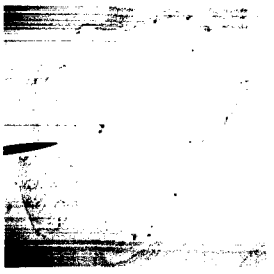
Canon PowerShot SD950 IS, 1/320, f/9,0, 9,57mm, ISO 200



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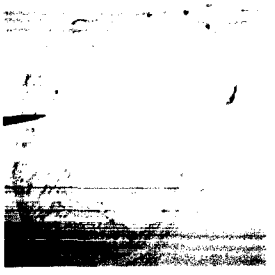

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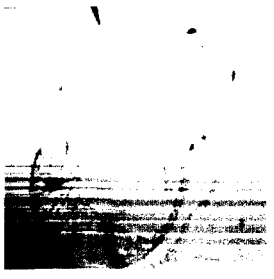

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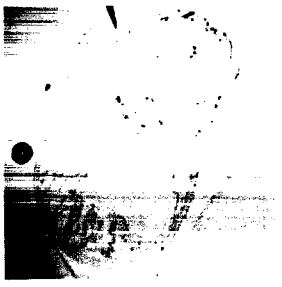
Canon PowerShot SD950 IS, 1/320, f/9,0, 9,57mm, ISO 200



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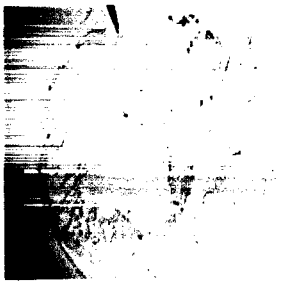

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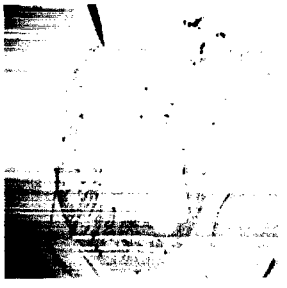

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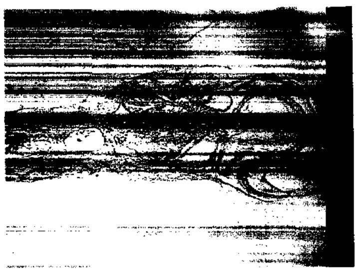
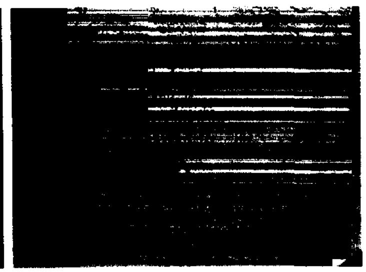
SUPPLEMENT 4 - H6-8 EMBRYO SERIAL SECTIONING IMMUNOSTAINING



Canon PowerShot SD950 IS, 1/60, f/4,0, 13,68mm, ISO 200



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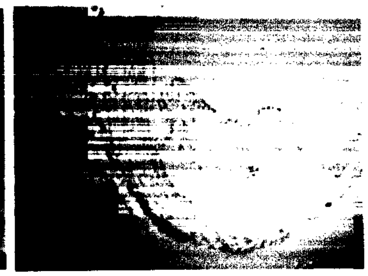

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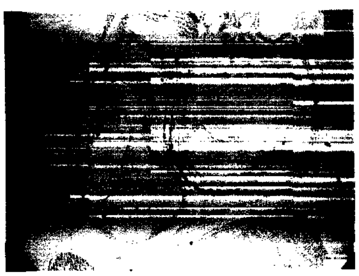
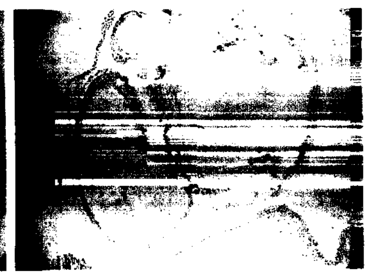


Canon PowerShot SD950 IS, 1/30, f/4,0, 16,29mm, ISO 320

Canon PowerShot SD950 IS, 1/30, f/4,0, 16,29mm, ISO 320



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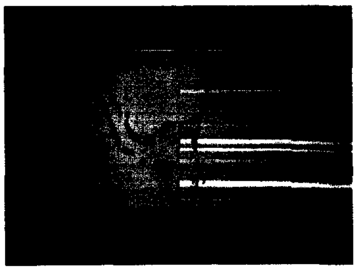
Canon PowerShot SD950 IS, 1/30, f/4,0, 16,29mm, ISO 320

Canon PowerShot SD950 IS, 1/30, f/4,0, 16,29mm, ISO 320

Canon PowerShot SD950 IS, 1/30, f/4,0, 16,29mm, ISO 320

Canon PowerShot SD950 IS, 1/30, f/4,0, 16,29mm, ISO 320

SUPPLEMENT 5 - H6-9 EMBRYO SERIAL SECTIONING IMMUNOSTAINING



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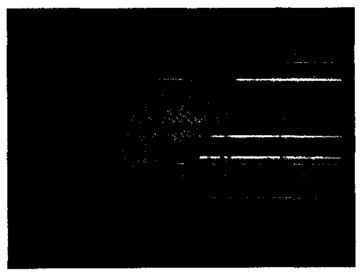
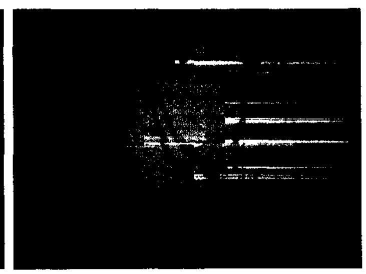
Canon PowerShot SD950 IS, 1/320, f/4,0, 13,68mm, ISO 200



Canon PowerShot SD950 IS, 1/400, f/4,0, 13,68mm, ISO 200



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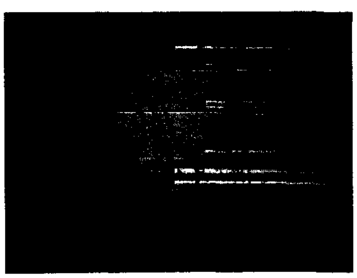

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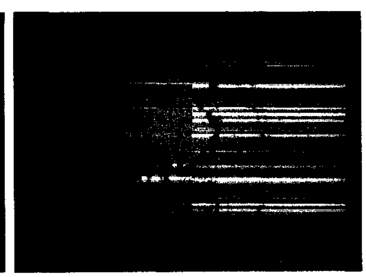

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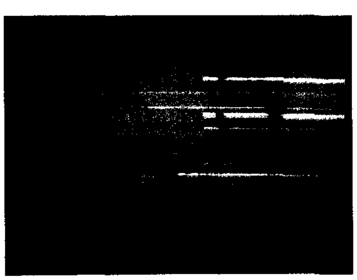

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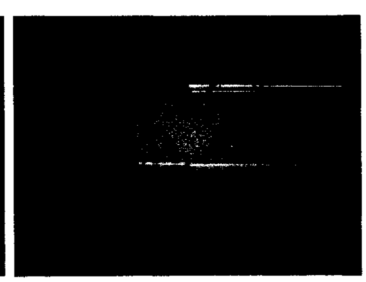

Canon PowerShot SD950 IS, 1/320, f/4,0, 13,68mm, ISO 200

Canon PowerShot SD950 IS, 1/320, f/4,0, 13,68mm, ISO 200

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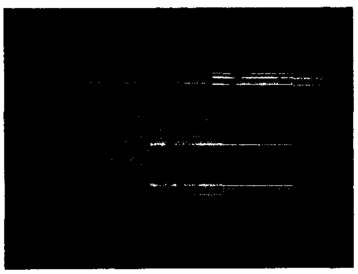
Canon PowerShot SD950 IS, 1/400, f/4,0, 13,68mm, ISO 200



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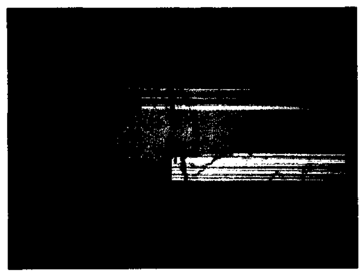

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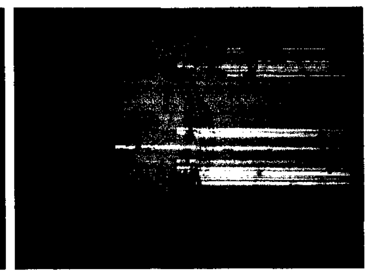

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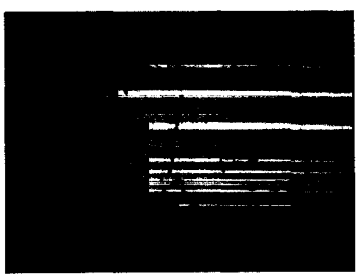

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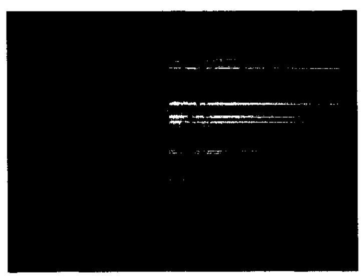

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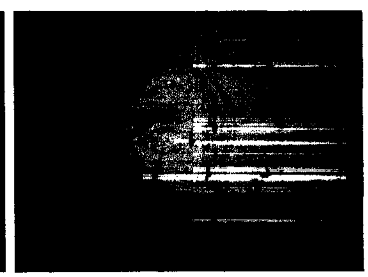

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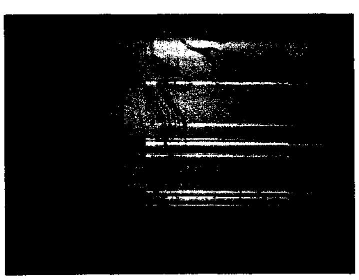
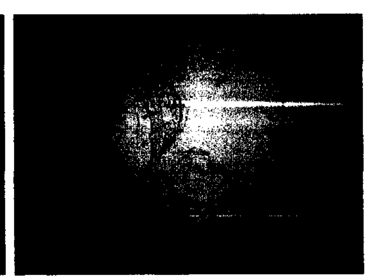


Canon PowerShot SD950 IS, 1/400, f/4,0, 13,68mm, ISO 200

Canon PowerShot SD950 IS, 1/400, f/4,0, 13,68mm, ISO 200



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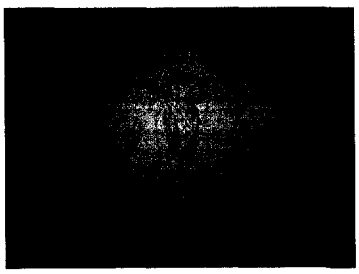

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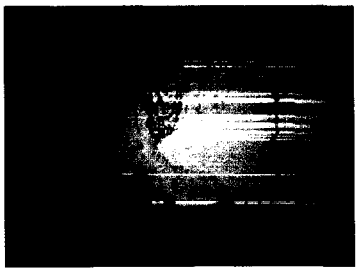

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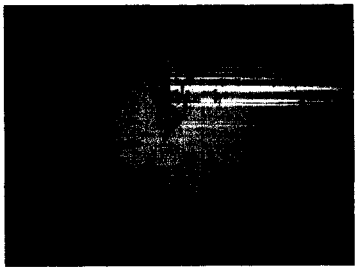

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Canon PowerShot SD950 IS, 1/320, f/4,0, 13,68mm, ISO 200