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PART II.

NOVEL FUNCTIONS FOR HEDGEHOG SIGNALING

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# CHAPTER 7.

## The Hedgehog Morphogen in Cardiovascular Disease

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### SUMMARY

**In this review we focus on the basic biology of the important developmental Hedgehog (Hh) protein family, its general function in development, pathway mechanisms, and gene discovery and nomenclature. Hh function in cardiovascular development as well as recent findings concerning Hh signaling in ischemia models is discussed in more detail and future perspectives are proposed. In light of the recent discovery of Hh transport by insect lipophorin, we also hypothesize a role for LDL in mammalian Hh transport, creating a surprising role for LDL in cardiovascular disease.**

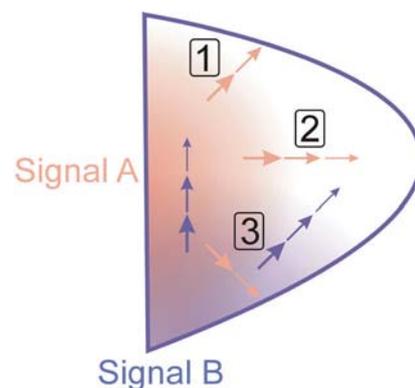
### A SHORT INTRODUCTION TO MORPHOGENS

A cell located in its surrounding tissue -either in a developing organism or adult tissue- has no “map” or any other autonomous clue about its position. Consequently, the cell does not know where it is, what it has to do, how to change its expression pattern, when to proliferate, where to migrate to, or even when to die. For structures as complex as the vasculature, informing cells about their required action is not only a necessity but also a challenge.

Two main mechanisms inform cells about their position. By cell-cell interactions, cells receive information concerning their position from neighboring cells. Alternately, gradients of signaling proteins called “morphogens” (reviewed in (Ashe and Briscoe, 2006)) diffuse through tissue over time. In the illustration for the hypothetical morphogens Signal A & Signal B (Figure 1), cell 1 has a high concentration of signal protein A in its local environment, whereas cell 2 senses a lower concentration. Based on the simple information conveyed by a single morphogen gradient, these cells are already destined to different fates. This mechanism suffices in the de-

veloping neural crest, which gives rise to a variety of tissue, including the aortic arch. (Payne et al., 1995) In addition to sensing a certain concentration of Signal A, cell 3 also senses Signal B, and is able to act accordingly. In a nutshell, the interplay between different morphogen gradients has complex results – for instance, determining how extremities and digits are formed. However, in some situations, the mere presence of a morphogen rather than a gradient is enough for proper patterning or tissue repair. While morphogen gradients are important in developing tissues, it seems that they are not that important in stable adult cells, where cell-cell interactions predominate in determining cell fate.

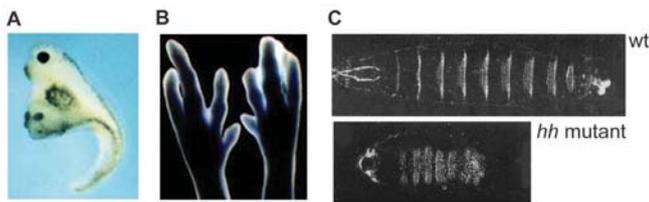
In humans, four families of morphogens are critical for providing almost all positional information. Hence the expression “morphogenetic code” is used to describe the combined action of the four morphogen gradients that



**FIGURE 1. GRADIENTS OF MORPHOGENS PATTERN TISSUE BY INFORMING CELLS ABOUT THEIR POSITION**

ARROWS INDICATE DIRECTION OF DIFFUSION IN DEVELOPING TISSUE (LIMB BUD IN THIS CASE). CELLS RECEIVING DIFFERENT CONCENTRATIONS OF THE TWO PROTEINS ARE NUMBERED AND THE ARROWS INDICATE THE DIRECTION OF MORPHOGEN DIFFUSION, THE “SIGNAL” LABELS INDICATE THE SOURCE OF THE GRADIENTS.

provide positional information to the differentiating cell. The first of these families is the wingless/Int (Wnt) family of extracellular glycoproteins, important in axis determination in the developing embryo (Sokol et al., 1991) (see Figure 2 A), heart valve formation (Hurlstone et al., 2003), and colorectal cancer (through activating mutations in this signaling pathway, Taipale and Beachy, 2001). The second family is the group of hormones belonging to the transforming growth factors  $\beta$  (TGF $\beta$ )/bone morphogenetic proteins (BMP)/Activins family, involved in a plethora of events including immunosuppression, ovulation, and induction of apoptosis in the membranous sheets between the various digits during gestation (Graff, 1997; Zou and Niswander, 1996, see Figure 2B). Third, the fibroblast growth factor (FGF) family, which is important in inducing stem cell proliferation in hematopoiesis and developing the blood islands during embryogenesis (Rissau and Flamme, 1995). Finally, the family of Hedgehog (Hh) proteins (Bijlsma et al., 2004) (Figure 2C), which consists of highly similar small secreted proteins that function in the developing organism, adult physiology, and tumorigenesis, all discussed in more detail below.



**FIGURE 2. FUNCTIONS OF DIFFERENT MORPHOGENS**

(A) THE WNT FAMILY OF EXTRACELLULAR PROTEINS FUNCTIONS IN BODY AXIS DETERMINATION, AS SHOWN HERE IN A CLAWED FROG (*XENOPUS LAEVIS*) EMBRYO, IN WHICH AN ADDITIONAL SOURCE OF WNT IS INJECTED (WNT-1 RNA), LEADING TO THE FORMATION OF A SECOND HEAD (FIGURE TAKEN FROM (SOKOL ET AL., 1991)).

(B) TGF $\beta$  AND BMP PROTEINS INITIATE APOPTOSIS TO SEPARATE THE DIGITS. SHOWN HERE ARE DEVELOPING CHICK LEGS, THE RIGHT ONE INFECTED WITH A DOMINANT NEGATIVE BMP RECEPTOR, SHOWING DEFECTIVE DIGIT SEPARATION (FIGURE TAKEN FROM (ZOU AND NISWANDER, 1996)).

(C) THE DENTICLES PATTERN IN FRUIT FLY EMBRYOS SHOW THE DISTINCT PHENOTYPE OF HEDGEHOG (HH) MUTANTS COMPARED TO WILD TYPE (WT). FIGURE ADAPTED FROM NUSSLEIN-VOLHARD C, WIESCHAUS E. 1980 NATURE 287:795–801 WITH PERMISSION OF NATURE PUBLISHING GROUP.

Some features unique in biology have been found for the Hh proteins and therefore, Hh draws considerable attention from the research and medical community. It is particularly fascinating that the mature protein is derived from autocatalytic cleavage (i.e. the protein cleaves itself) of a precursor protein, followed by the addition of a cholesterol and a palmitoyl group (Pepinsky et al., 1998; Porter et al., 1996). Hh is the only known sterolated protein in the animal kingdom

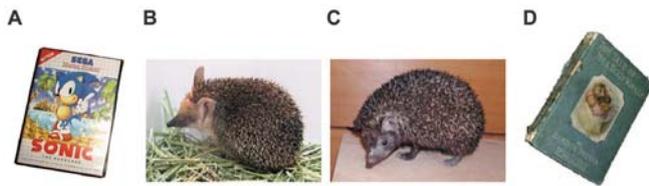
and Hh proteins are one of the few palmitoylated proteins that are secreted (another example of secreted palmitoylated proteins are the Wnt proteins). Not unexpectedly, the lipophilic moieties limit the diffusion capacity of Hh in the aqueous medium that surrounds cells, leaving the mechanisms by which Hh is distributed throughout tissues subject to fierce debate.

## DISCOVERY OF THE HEDGEHOG MUTANT IN *DROSOPHILA MELANOGASTER* AND NEW HOMOLOGS

In wild type fruit fly (*Drosophila melanogaster*) larvae, a band of denticles runs across the anterior half of each segment, whereas the posterior half is smooth (naked cuticle). In screening for mutations that affect the segmental pattern of the fruit fly larvae, Nüsslein-Volhard and Wieschaus discovered a group of mutations that affected the patterning within segments, but left the number of segments unaltered (Nüsslein-Volhard and Wieschaus, 1980). One of these segment polarity mutants caused denticles to occur not only on the anterior, but also on the posterior half of the segments, covering the back of the larvae with a continuous lane of bristles as shown in Figure 2C. Nüsslein-Volhard and Wieschaus were awarded the Nobel Prize for this discovery in 1995.

In 1993, a joint effort of three research groups (labs of McMahon, Tabin and Mohler) resulted in reporting the first vertebrate Hh genes (Echelard et al., 1993; Krauss et al., 1993). Echelard used the cloned fruit fly Hh to identify three genes homologous to fruit fly Hh in mouse and chicken ((Echelard et al., 1993) and personal communication with Y. Echelard, PhD, unpublished data, 2003). As shown in Figure 3, these homologues were comically termed Sonic Hh, after a Sega arcade game character introduced in 1990, Desert Hh, after an Egyptian species of hedgehog (*Hemiechinus auritus*), and Indian Hh, a hedgehog species endemic to the Indian peninsula (*Hemiechinus micropus*).

The name Sonic Hh was proposed by Robert Riddle and Randy Johnson, for it seemed to be the “all powerful” of the Hh homologs, just like Sonic the Hedgehog is all powerful in his hit arcade game. While the playful nomenclature for the Hh homologs is testimony of geneticists’ sense of humor, the number of Hh species to name Hh homologs seems depleted. One research group has now chosen to use “quahog” as homolog name (Hao et al., 2006) in nematodes, and in fish, “Tiggy-Winkle” Hedgehog is known (Ekker et al., 1995). Although the above-mentioned proteins were found to share great homology, they did not colocalize in the developing organism, and it seems they have varying functions regardless of their similarity.



**FIGURE 3. ETYMOLOGY OF SOME HH HOMOLOGS**

(A) THE PREDOMINANT HH HOMOLOG IN MAMMALS, SONIC HH, WAS NAMED AFTER AN ARCADE GAME CHARACTER “SONIC THE HEDGEHOG”.

(B) THE DESERT HH HOMOLOG WAS NAMED AFTER A HEDGEHOG SPECIES FROM AFRICA.

(C) THE INDIAN HEDGEHOG SPECIES FROM THE INDIAN PENINSULA, GAVE ITS NAME TO ANOTHER HOMOLOG.

(D) ANOTHER FICTIONAL HEDGEHOG CHARACTER “MISS TIGGY-WINKLE” WAS CHOSEN TO TERM A FISH HH HOMOLOG.

### FUNCTION OF THE HEDGEHOG PROTEINS IN CARDIOVASCULAR DEVELOPMENT

Long after its discovery as a segmental patterning protein in fruit fly, the Hh protein family is continuously found to be involved in new processes, many of which were previously attributed to other proteins or compounds, or were simply unexplained. *In situ* hybridization and immunohistochemical studies showed that mammalian Hh homologues displayed intricate expression patterns during development as well as in adult organisms, especially in the vascular system. Especially interesting to the scope of this review is the involvement of Hh in the morphogenesis of the heart and blood vessels. A clue to this involvement was the ability of Shh to induce VEGF and angiopoietins in human fibroblasts (Pola et al., 2001), but a more definite answer came from studies in mice deficient for Hh pathway components. Seemingly, Hh proteins (mainly Ihh and Shh) are of pivotal importance to vascular remodeling in the yolk sac of the developing mouse (excellently reviewed in (Byrd and Grabel, 2004)). In the mesoderm, so-called blood islands, the first hallmarks of vascular structures in the yolk sac, are formed in response to fibroblast growth factor (FGF) (Risau and Flamme, 1995) (although *in vitro*, a requirement for Smo and Ihh was found). These blood islands are lined by angioblasts, cells that will later form the endothelium, and filled with hematopoietic cells, primitive erythrocytes. These blood islands then fuse to a honeycomb-like network (the primary capillary - or vascular plexus) and the angioblasts differentiate to become endothelium. In *Smo* mutant mice, the activating receptor for the Hh pathway, development stops here and there is no further development of the vasculature (Byrd et al., 2002). The capillary plexus in mice genetically deficient for Hh proteins will be remodeled to early vessels, however these are clearly smaller and less organized than in the wild type vasculature. The responsiveness to Hh of en-

dothelial cells or endothelial precursor cells themselves is not yet clear. Although most groups show unresponsiveness to Hh for these cells and suggest the action of intermediate cells in translating the Hh signal to for instance VEGF or angiopoietins, (Byrd et al., 2002; Pola et al., 2003) others have been able to induce capillary morphogenesis in endothelial cells by Hh *in vitro* (Kusano et al., 2004).

The development of the heart starts very early in the developing embryo, as progenitor cells in the mesoderm form the primitive cardiac tube. The straight tube then undergoes rightward looping and subsequently, the atrial and ventricular chambers appear and mature. Due to the function of Hh proteins in determining left/right asymmetry (Levin et al., 1995; Meyers and Martin, 1999), heart tube looping is critically dependent on proper Hh processing (Tsukui et al., 1999). In *Shh*<sup>-/-</sup> mice embryos, numerous defects in cardiac development are seen, which are briefly summarized in the following section.

Although the atrial and ventricular chambers are formed (suggesting completed heart looping) in *Shh* mutant mice, they differ from wild type in many aspects. For instance, a single common atrium is seen and a pulmonary valve is absent. The right ventricle is reduced, whereas the left ventricle is extended. Furthermore, the heart is positioned on the left side of the thorax, and the shape of the heart indicates a laterality defect. Surprisingly, although heart looping is delayed and incomplete in these animals, the direction of heart looping is unchanged. In *Gli2*<sup>-/-</sup>;*Gli3*<sup>+/-</sup> double mutant mice (thus lacking 2 out of 3 Hh transcription factors), disturbed cardiac development very similar to that found in *Shh* mutants is observed (Kim et al., 2001). In *Shh* and *Ihh* double mutants as well as *Smo* mutants, no heart looping and successive heart development was observed at all. As these mice are arguably most deficient in Hh signaling, the severe phenotype argues for an absolute requirement for Hh signaling in cardiac development (Zhang et al., 2001). Mice mutant for the inhibitory Hh pathway component *SuFu* showed inverted heart looping, indicating that heart looping is not only affected by diminished Hh function, but also by aberrantly increased pathway activity (Cooper et al., 2005).

Later in cardiac development, cells from the neural fold (neural crest cells or NCCs) are recruited to form the aortic arches, the cardiac outflow tract, and the proximal great vessels. In addition to the anomalous cardiac development described above, *Shh*<sup>-/-</sup> mice embryos show defects in NCC localization and increased cell death, leading to the absence of the ductus arteriosus, abnormal subclavian arteries, and a single midline carotid artery. It is remarkable that *Shh* signaling is pivotal in two different events in cardiac development, testimony to its relevance in the developing organism.

## HEDGEHOG IN ISCHEMIA

As mentioned previously, Hh proteins have long been known to act in the developing organism, and to remain active in adult physiology (e.g., in the histostability of the gastrointestinal tract) (van den Brink et al., 2001). Among Hh-regulated processes in adults, revascularization of ischemic tissue is of utmost clinical importance. The previously described Hh signaling in the development of certain elements of the cardiovascular system pointed to a vascularizing or patterning role for Shh. In recent years, this vascularizing role seems to be only one of the beneficial effects that the Hh pathway can confer to ischemic tissue.

Two recent papers of Pola *cum suis* (Pola et al., 2003; Pola et al., 2001) established a strong role for Hh signaling in adult cardiovascular pathophysiology. They showed that Hh mediates a profound upregulation of target genes involved in angiogenesis, indicating the responsiveness of adult tissue to Hh. Remarkably, VEGF and the angiopoietins Ang-1 and -2 were simultaneously upregulated in response to Shh, and concomitantly Shh was found to be a potent inducer of vascularization in a cornea model. It is important to note that following *in vitro* experiments, the authors found that endothelial cells do not respond to Hh, and -as abovementioned for the developing embryo- suggest an intermediate action of mesenchymal cells. As previously mentioned, Byrd and co-workers confirmed that Hh responsiveness is confined to mesothelial and smooth muscle cells (Byrd et al., 2002). The authors extended their findings and managed to salvage ischemic hind limbs in mice by injecting Shh in the afflicted muscle. Later, the specificity and mechanism behind this salvage was further elucidated and the Hh response to ischemia was found to be an endogenously occurring process (Pola et al., 2003).

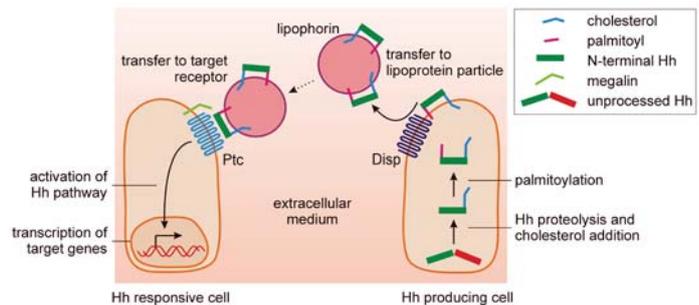
In an elegant experimental setup, Kusano (Kusano et al., 2005) recently demonstrated that intramyocardial gene transfer of Shh promoted recovery and preservation of left ventricular function in both acute and chronic myocardial ischemia by enhanced neovascularization and recruitment of bone marrow-derived endothelial progenitor cells. Reduced fibrosis and cardiac apoptosis was observed after Shh gene transfer. In this myocardium model, the endogenous activation of Shh in ischemia was again confirmed, suggesting that the observed salvage by injecting Shh-DNA is not an artificially created situation, but rather an augmentation of a naturally occurring phenomenon. The obvious potential of endogenous Hh to induce significant tissue alterations and cell recruitment raises questions as to the involvement of Hh in the development of (rare) angio-, fibro- or rhabdomyosarcomas (Hahn et al., 1998).

In cerebral ischemia, a positive role for Hh-mimicking molecules has also been shown in limiting the damage

caused by artificial vessel occlusion in rats (Nobel Conference, Stockholm, 2004) (Briscoe and Therond, 2005). Together, these data strongly support the notion that Hh aids in the rescue of ischemic tissue, especially in its revascularization. This Hh-dependent revascularization probably reflects a physiological response to ischemic stress, although what exactly drives this Hh response (e.g. hypoxia or inflammation) is not yet known. Many signals that are generated in ischemic tissue, ranging from cytokines to acidity might trigger Hh expression. Also, we do not know which specific areas in the ischemic tissue activate Hh expression, the degree of tissue damage still considered “salvageable” enough for the Hh pathway to function, or whether the complex Hh pathway remains intact in damaged cells.

## LIPHOPHORIN CARRIES THE HEDGEHOG PROTEIN

One of the major questions remaining with respect to Hh signal transduction today is the mechanism that underlies the distribution of the morphogen itself. Hh is a highly unusual intercellular messenger, as the protein is both sterolated (Porter et al., 1996), and palmitoylated (Pepinsky et al., 1998) and therefore extremely hydrophobically modified. Hence, its diffusion capacity is strongly limited in the aqueous medium that surrounds cells. Hh is, however, capable of long-range signal transduction and various models have been put forward to explain Hh distribution in tissues. The most simple of these models is the formation of Hh multimers (Feng et al., 2004), in which the hydrophobic groups of several Hh molecules are arranged in such a way that the protein complex becomes



**FIGURE 4. LIPHOPROTEIN MEDIATED HH TRANSPORTATION**

AFTER SYNTHESIS, AUTOCATALYTIC PROTEOLYSIS AND LIPHOPHILIC MOIETY ADDITION, THE HH PROTEIN IS NOW PRESUMED TO BE TRANSFERRED TO A LIPHOPROTEIN PARTICLE, LIPHOPHORIN IN *DROSOPHILA*. THE TRANSFER OF STEROLATED HH TO LIPHOPHORIN MIGHT BE MEDIATED BY DISP. BY UNKNOWN MECHANISMS, PROBABLY INVOLVING HEPARAN SULFATE PROTEOGLYCAN (HSPCs), THE PARTICLES TRAVEL A CERTAIN DISTANCE THROUGH THE TISSUE AND REACH A TARGET -HEDGEHOG RESPONSIVE- CELL. ENDOCYTOSIS OF THE ENTIRE PARTICLE-BOUND HEDGEHOG OR PERHAPS JUST DOCKING TO THE CELLS SURFACE IS PROBABLY MEDIATED BY MEGALIN, THE MAIN RECEPTOR FOR HEDGEHOG HOWEVER BEING PTC.

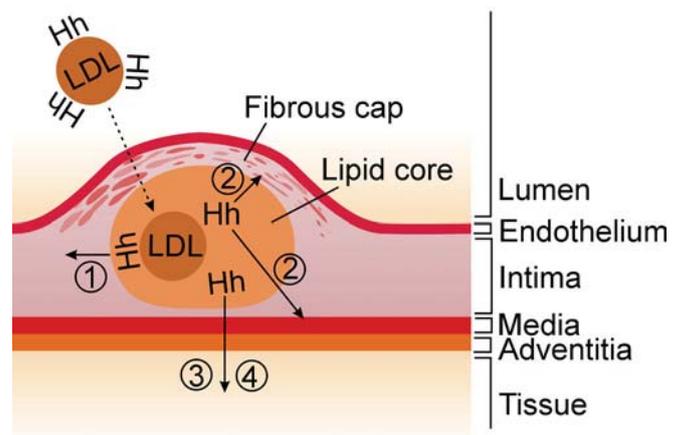
soluble in a polar medium. Another model is built on the suggestion that cells pass on Hh, by means of endo- and exocytosis cycles that presumably involve the known receptor for Hedgehog, Ptc and a homolog of Ptc, dispatched (Disp) (Briscoe and Therond, 2005). Tanaka *et al.* recently described transportation of Hh by means of “nodal vesicular parcels” or NVPs (Tanaka *et al.*, 2005). In this model, the NVPs containing Hh are transported through the ventral node fluid by ciliary movement. Recently however, an even more appealing way of transporting the amphipathic Hh molecule throughout the body has been proposed (Panakova *et al.*, 2005).

Very similar to the vertebrate circulation, in which cholesterol is transported by lipoproteins, in a recent study by Panakova *et al.* (2005), *Drosophila* Hh was found to be carried by the insect equivalent of LDL, lipophorin. The authors showed that glycosylphosphatidylinositol (GPI)-anchored and sterolated proteins, amongst which some well known morphogens, colocalize with lipophorin and that proper patterning by these morphogens requires lipophorin. This lipophorin-bound Hh is carried away from the *Drosophila* fat-body where lipophorin is generated, to signal in the imaginal discs (summarized in Figure 4).

Interfering with this lipophorin-dependent transport mechanism severely restricts the range of Hh signaling. This remarkable finding creates new insights into the possible action of Hh in physiology; Hh loading on lipophorin implies a seemingly endless diffusion capacity for Hh. Although the authors speculate on transport through tissue, transport of Hh together with the hemolymphatic circulation in insects seems a logical implication of the finding described by Panakova *et al.* Thus, local Hh production, ironically by virtue of its hydrophobicity, loaded on lipophorin may affect tissue far away from the source of morphogen production. Interestingly, the mammalian homologues of *Drosophila* Hh, i.e. Sonic Hh, Indian Hh, and Desert Hh, share the extensive posttranslational sterolation and palmitoylation with *Drosophila* Hh. Consequently, these mammalian Hh homologues are also partly hydrophobic molecules (Jeong and McMahon, 2002). It seems plausible that in mammalian systems Hh is loaded on the mammalian equivalent of lipophorin, LDL (Van Hoof *et al.*, 2002). This point was also not lost by Panakova *et al.* (2005), who highlighted that megalin, a protein with strong homology to LDL receptors, has been shown to mediate endocytosis of Hh independent of Ptc and that megalin seems to be necessary for normal Hh signaling *in vivo*. This mechanism would provide the means to “catch” LDL-bound Hh specifically. If LDL would be a source of Hh, however, this could change our view on the role of LDL in cardiovascular disease.

## HEDGEHOG TURNS LIPOPROTEINS INTO JANUS-FACED PARTICLES

One of the major contributors to human cardiovascular disease and ischemic stress is atherosclerosis, characterized by atherosclerotic depositions in the medium and larger size arteries (Orford *et al.*, 2000). Although the exact cause of progression of atherosclerosis is unknown, a negative role for lipid transport to the intima of the arteries by LDL has been well established. The accumulation of these lipids and subsequent progression of the atherosclerotic plaque may cause its rupture or fragmentation leading to thrombosis causing, amongst others, tissue ischemia. As it is generally accepted that LDL is the carrier of cholesterol from hepatic to peripheral tissues and that an excess of LDL causes atherosclerosis, LDL has since long been considered the “bad” cholesterol carrier. The lipophorin Hh transport mechanism as found by Panakova *et al.* (2005) however suggests that LDL in human circulation could function in a similar way, transporting Hh through the circulation, accumulating in plaques and providing a natural signal for revascularization.



**FIGURE 5. POSSIBLE ROLES FOR Hh SIGNALING IN AN ATHEROSCLEROTIC PLAQUE**

WHEN THE INTIMA AT THE LOCATION OF ATHEROSCLEROTIC LESION GROWS, LIPIDS ACCUMULATE IN THE SO-CALLED “LIPID CORE”. AS LDL PARTICLES INCORPORATE LIPIDS INTO THE PLAQUE, THE LIPID CONTENT IN THE INTIMA GROWS. POSSIBLY, Hh IS TRANSFERRED FROM THE LDL PARTICLES IN THE LIPID CORE TO THE INTIMA AND SURROUNDING TISSUE. SOME OF THE ANTICIPATED CELLULAR PROCESSES THAT COULD BE AFFECTED BY THIS Hh ARE 1) MODULATION OF T-LYMPHOCYTE CELL CYCLE PROGRESSION (LOWREY *ET AL.*, 2002), ACTIVATION AND CYTOKINE PRODUCTION (STEWART *ET AL.*, 2002); 2) PROLIFERATION AND MIGRATION OF SMOOTH MUSCLE CELLS (KUSANO *ET AL.*, 2004); 3) REVASCULARIZATION OR OTHER REGENERATIVE PROCESSES INDUCED IN ADJACENT TISSUE. (POLA *ET AL.*, 2001) 4) RECRUITMENT OF STEM CELLS BY Hh AS KNOWN FOR MYOCARDIAL ISCHEMIA. (KUSANO *ET AL.*, 2005) ALTERNATIVELY, Hh COULD REMAIN IN THE PLAQUE DUE TO ITS HYDROPHOBICITY AND INDUCE VASCULARIZATION THEREIN, A HARMFUL PROCESS. (MCCARTHY *ET AL.*, 1999)

Thus lipoproteins could function as both a risk factor for cardiovascular disease by causing atherosclerotic plaques, but also as a carrier for Hh-dependent revascularization and tissue salvage. If this hypothesis is true, inhibitors of LDL cholesterol loading (that diminish LDL cholesterol content, but not cholesterol synthesis) would have superior therapeutic potential compared to generic sterol biosynthesis inhibitors that also impair Hh bioactivity (which is highly dependent on its lipid modifications). These cholesterol loading inhibitors diminish the cholesterol content on LDL but do not affect Hh activity on LDL.

Hence, an interesting change of paradigm might emerge for LDL. No longer would LDL be merely bad news in the progression of atherosclerotic plaques, as it also conveys a revascularization or patterning signal in the form of Hh. In that case, Hh containing LDL would serve an intrinsic repair mechanism by which the body tries to limit the damage done by the plaque. Based on known effects of Hh in various model systems, it is attractive to hypothesize that Hh in this context plays its beneficial role via; 1) proliferation (Kusano et al., 2004) and perhaps migration of smooth muscle cells, perhaps also into the intima. 2) modulation of T-lymphocyte cell cycle progression, activation, and cytokine production (Stewart et al., 2002). 3) induction of revascularization or other regenerative processes in adjacent tissues (Pola et al., 2001). See also Figure 5.

Together, these data suggest that Hh loading on lipoproteins may force us to abandon the current one-dimensional LDL doctrine in vascular disease. Obviously, however, one should realize that significant differences may exist between vertebrates and arthropods with respect to Hh signal transduction and that this might also prove true for the transport of Hh by lipoproteins.

## FUTURE PERSPECTIVES

Once the practicalities that surround the different variants of gene therapy have been overcome, it is very likely that Shh-DNA will provide treatment options for patients that survived an initial myocardial ischemia. Should DNA transfer remain a problem in the future because of the potential danger of horizontal transfer or genome incorporation, an interesting role for RNA-based therapies might emerge. As RNA is very unlikely to be reverse-transcribed after transfer, issues with horizontal gene transfer or chromosome integration are avoided.

The current problems with RNA stability and delivery, however, pose challenges that preclude application at this time. Before wide-scale injection of Shh protein or DNA would be feasible, problems involving costs and the intricacy of uniformly injecting compounds in the myocardium must be

overcome. While the development of chemically synthesized small-molecule agonists for the Hh pathway may provide a thrust forward, their distribution could prove too systemic in practice. Using a molecule that specifically recognizes ischemic tissue coupled to a Hh agonist can provide us with a specific, potent, and relatively affordable therapeutic option (resembling the aptamer approach developed for specific targeting of cancer cells (Farokhzad et al., 2006)). Targeting Smo rather than Ptc with small molecules, for instance using purmorphamine, eliminates any Smo-independent effects. Also, this should further enhance specificity as well as potency, as it eliminates the inhibitory actions on Smo of yet unidentified Smo-inhibitory proteins (Ptc2 is known for instance (Motoyama et al., 1998), but others might arise). Evident from the paper of Kusano (Kusano et al., 2005) was the involvement of Hh in enhancing myocardial influx of bone marrow-derived progenitor cells. As this naturally occurring population of cells finds its way to the damaged myocardium, they could perhaps confer specificity to the action of systemically administered Hh. Of course, this would not be feasible when Hh is, in fact, the recruiting factor for these cells. Also, bone marrow-derived stem cells have been implicated in tumorigenesis (for instance, in the stomach (Houghton et al., 2004)); so overly enhanced proliferation of such circulating stem cells might not necessarily prove beneficial in the long run.

In summary, we predict that through intense research efforts our understanding of Hh pathway mechanisms and the participation of the Hh pathway in cardiovascular development will continue to develop. Together with progress in technologies such as gene therapy and -diagnostics, this will aid in applying our knowledge and offer us a means to combat cardiovascular disease in adult as well as the developing child.

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