1993). In SMCs, TGF-β is known to inhibit proliferation induced by growth factors (Owens et al. 1988). As described above, TGF-β also shows a potent matrix-depositing activity in the vascular wall. These biological properties of TGF-β seem to affect each step of atherogenesis in a favorable manner to give a stable plaque phenotype rich in matrix with limited accumulation of inflammatory cells.

In vivo experimental results support the anti-atherogenic or plaque-stabilizing function of TGF-β. Mice heterozygous for TGF-β1 gene showed endothelial activation and lipid lesion formation, when fed on high fat diet (Grainger et al. 2000). Inactivation of TGF-β signal by systemic administration of neutralizing antibody or soluble TβR-II in atherosclerosis-prone apoE-knockout mice resulted in vascular lesions with a higher ratio of inflammatory cells and reduced fibrosis compared to the controls (Lutgens E et al. 2002, Mallat et al. 2001). Conversely, treatment with an anti-estrogen tamoxifen increased serum TGF-β levels and suppressed the formation of aortic lesions in mice (Grainger et al. 1995b). More recently, it was shown that dominant-negative TβR-II specifically expressed in T-cells to abrogate TGF-β signaling gave "unstable-like" plaque phenotype in both apoE- and LDL receptor-knockout mice (Gojova et al. 2003, Robertson et al. 2003). The results indicate the prominent role of TGF-β function in T-cells for its anti-atherosclerotic activity.

These findings are in line with clinical observations that low blood levels of active TGF-β associates with the severity of vascular disease, suggesting a protective effect of TGF-β against atherosclerosis in human (Grainger et al. 1995a, Stefoni et al. 2002). Altogether, TGF-β seems to have plaque-stabilizing

potential in general. But when excessively activated, such as upon vascular injury, it may facilitate restenosis through accumulation of matrix and constrictive remodeling.

Disruption of Smad3-dependent TGF-β signal enhances neointimal hyperplasia with reduced matrix deposition upon vascular injury

In contrast to the accumulating knowledge on the role of TGF-β in restenosis and atherosclerosis, the precise function of individual signaling molecules for TGF-β in vascular disease remains unclear. Therefore, we examined the mice null for Smad3 *in vivo* and *in vitro* to clarify the function of Smad3-dependent signaling in vascular response to injury (Kobayashi et al. 2005).

Femoral arteries of Smad3-null mice showed significant enhancement of neointimal hyperplasia compared to those of wild-type mice (Fig 3A, B) upon endothelial injury by photochemically-induced thrombosis method. Immunohistochemical examination revealed that neointima was exclusively composed of SMCs. Transplantation of Smad3-null bone marrow to wild-type mice did not enhance neointimal thickening, suggesting that vascular cells *in situ* play a major role in the response. Smad3-null neointima compared to wild-type showed a higher cell density with increased proliferative activity of SMCs. On the other hand, Masson's trichrome staining revealed significantly reduced extracellular collagen accumulation relative to total intimal area in Smad3-null artery (Fig.3C, D). These findings suggest that Smad3-deficiency causes neointimal lesions rich in SMC but scarce in matrix upon vascular injury.

In vitro, TGF-β inhibited serum-stimulated DNA synthesis of wild-type aortic SMCs with the maximal inhibition of 70%. However, growth of

Smad3-null SMCs was only weakly inhibited by TGF-β, indicating an essential role of Smad3 in TGF-β-mediated growth inhibition of vascular SMCs as reported in other cell types (Ashcroft et al. 1999, Datto et al. 1999). Unexpectedly, Smad3-null SMCs dose-dependently migrated towards TGF-β at least to a similar extent as wild-type cells, suggesting that non-Smad3 signal mediates TGF-β-induced chemotaxis in murine vascular SMCs. The finding differs from the previous report demonstrating an indispensable role of Smad3 in migration of monocytes and neutrophils towards TGF-β by (Aschcroft et al. 1999). In terms of matrix regulation, TGF- β increased the transcript levels of α2 type I collagen and tissue inhibitor of metalloproteinases-1, but suppressed expression and activity of MMPs in wild-type SMCs. In Smad3-null SMCs, TGF-β was inefficient in inducing collagen or suppressing MMPs giving a possible explanation for reduced extracellular matrix in Smad3-null neointima *in vivo*.

Role of endogenous Smad3 in vascular homeostasis: limiting neointimal hyperplasia?

Fig. 4 illustrates the possible mechanism underlying the enhanced neointimal hyperplasia in Smad3-null mice. Upon endothelial injury in the wild-type artery, endogenous Smad3 allows TGF-β to elicit growth inhibitory effect on intimal SMCs and to promote extracellular matrix accumulation, resulting in "healing" of vascular lesions with modest intimal thickening (Fig. 4A). When Smad3 is absent (Fig. 4B), SMCs are largely resistant to growth inhibitory control by TGF-β and thus undergo increased proliferation. On the other hand, impaired collagen synthesis as well as overall upregulation of matrix-degrading activity by TGF-β leads to reduced amount of matrix in the intima of

Smad3-null arteries. Degradation of matrix scaffold by MMPs enables cell movement and tissue reorganization (Lijnen et al. 1999, Galis and Khatri 2002). As mentioned earlier, the migratory capacity towards TGF-β is preserved in Smad3-null SMCs. Therefore, inability of TGF-β to suppress MMPs in null cells may facilitate migration from media to intima *in vivo* allowing further accumulation SMCs in intima. Moreover, since Smad3 is known to mediate anti-inflammatory activity of TGF-β in SMCs (Feinberg et al. 2004), activation of inflammatory genes in Smad3-null SMCs may also contribute to the accelerated neointimal formation.

Taken the results together, endogenous Smad3 is likely to have an effect to to limit the extent of neointimal hyperplasia through modulation of SMC functions in the process of restenotic vascular response.

Future perspectives

As described earlier, TGF-β ligand itself promotes intimal thickening in balloon injury models. Our findings that targeted deletion of Smad3, a major signal mediator of TGF-β, accelerates neointimal formation appear inconsistent with those observation on TGF-β. A possible explanation would be that our model differs from any other previous ones in the point it lacks Smad3 but not other TGF-β signal components. Non-Smad3 signals, such as MAP kinases, may act promotive on the lesion formation in vascular injury model (Fig. 5A). Although unlikely, difference in the method of endothelial injury, either balloon or thrombotic, should also be considered.

Finally, mice in which both the Smad3 and ApoE genes have been deleted show marked enhancement of atheromatous lesion resembling "unstable

plaque" compared to single ApoE knockout mice (Kobayashi et al. unpublished observation). Therefore, in this hypercholesterolemia model of atherosclerosis, Smad3 seems to at least in part mediate protective function of TGF-β (Fig. 5B). As judged from the results of Smad3-null mice, Smad3 plays a protective role in both injury/restenosis and hypercholesterolemia/atherosclerosis models. From a therapeutic point of view, it is of interest to know whether specific potentiation of Smad3 activity in the vascular wall leads to amelioration of neointimal hyperplasia and atheromatous lesions.

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Figure Legends

Figure 1. Schematic illustration of intracellular signal transduction pathways by TGF-β. Upon ligand-induced heteromeric complex formation and activation of type I and type II receptors, cytoplasmic signal transducers Smad2 and Smad3, classified as so-called receptor-activated Smads (R-Smad) are phosphorylated and heteroligomerize with Smad4, a common mediator Smad. The complex then translocate into to the nucleus, where it regulates expression of target genes. Smad7 binds to type I receptor, interferes with the phosphorylation of R-Smad and results in suppression of the signaling. Non-Smad signaling pathways, indicated as a broken arrow, are also reported. P indicates phosphorylated serine/threonine residues.

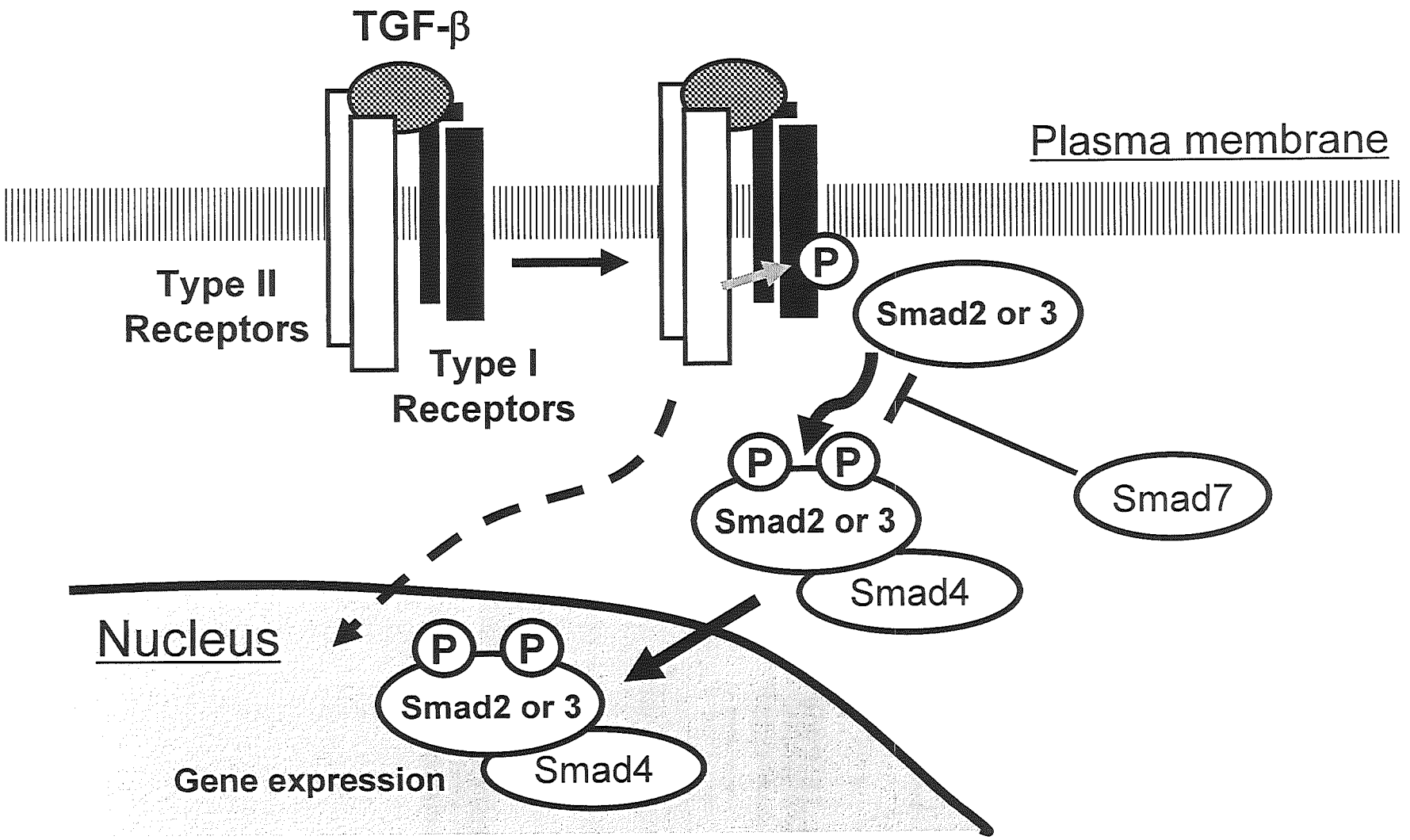
Figure 2. Major steps in atherosclerotic lesion formation and the putative effects of TGF-β on the each step. Both *in vitro* and *in vivo* evidence suggests that TGF-β inhibits activation of endothelial cells and intimal accumulation of inflammatory cells and smooth muscle cells. On the other hand, TGF-β promotes deposition of extracellular matrix.

Figure 3. Enhanced neointimal hyperplasia and reduced matrix deposition in the arteries of Smad3-null mice upon injury. Photomicrographs showing representative cross sections of hematoxylin/eosin-stained (A, B) and Masson's trichrome-stained (C, D) femoral arteries from wild-type (A, C) and Smad3-null (B, D) mice 3 weeks after endothelial injury by photochemically-induced thrombosis method. L, vascular lumen. Arrows indicate the positions of the

internal elastic lamina. Original magnification x200; bar = 50μm. (Reproduced with permission from Kobayashi et al. 2005, pp. 906 and 909, Copyright 2005, Lippincott Williams & Wilkins.)

Figure 4. Possible mechanism of enhanced intimal hyperplasia in the artery of Smad3-null mice after injury. In the presence of endogenous Smad3 (A), TGF-β inhibits intimal smooth muscle cell growth and induces deposition of matrix, modestly limiting the intimal thickening. In lack of Smad3 (B), intimal smooth muscle cells are resistant to growth inhibition by TGF-β. Increased proliferation of smooth muscle cells leads to enhanced intimal hyperplasia. Reduced matrix scaffold may also facilitate migration of smooth muscle cells from the medial layer.

Figure 5. Putative role of Smad3 in two distinct models of vascular disease.
A, In endothelial injury model, Smad3 has an inhibitory effect on formation of neointimal hyperplasia. The effect of TGF-β to promote neointimal hyperplasia is likely to be mediated by non-Smad3 signal. B, In hypercholesterolemia model, Smad3 at least in part mediates the anti-atherogenic function of TGF-β.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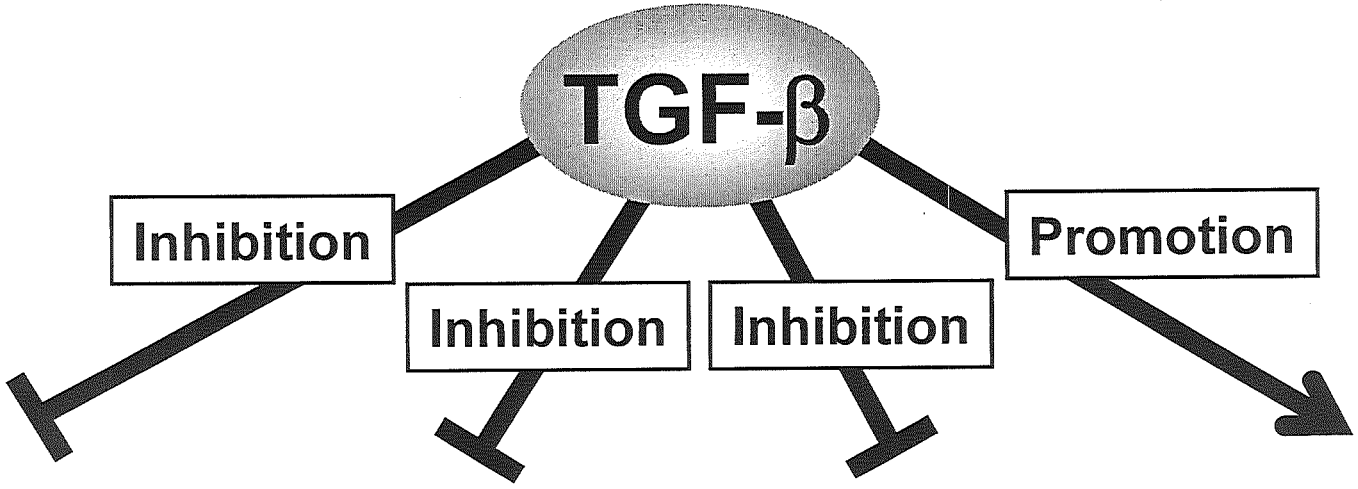
Type II
Receptors
Type I
Receptors
P
Smad2 or 3
P
P
Smad2 or 3
Smad4
Smad7
Nucleus
P
P
Smad2 or 3
Smad4
Gene expression



Endothelial Activation

Adhesion molecules ↑
Chemokines ↑
Cytokines ↑
Growth factors ↑

Macrophage and T-lymphocyte Accumulation

Chemokines ↑
Cytokines ↑
Growth factors ↑
Proteases ↑

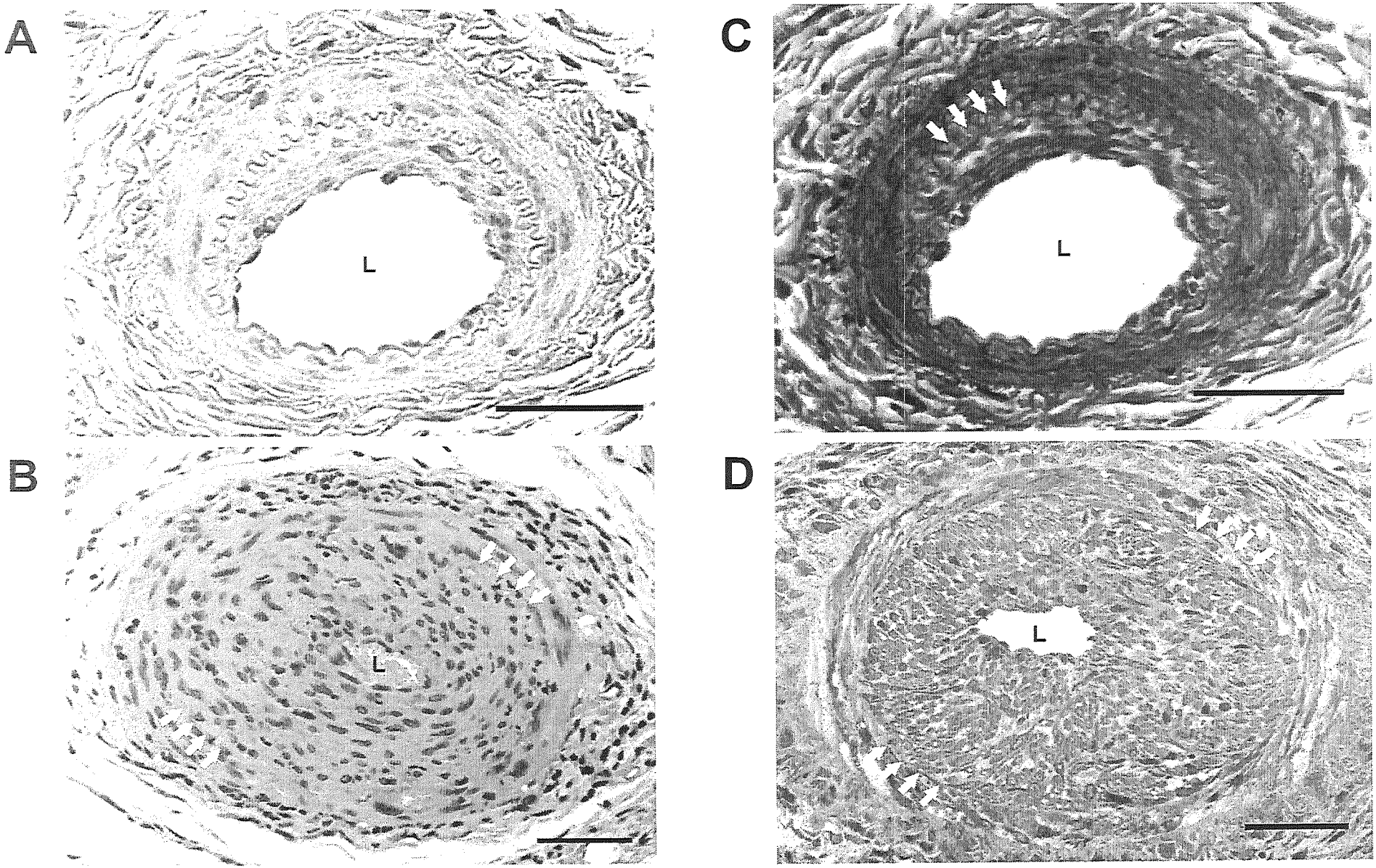
Smooth muscle cell Accumulation

Migration from intima ↑
Proliferation ↑
Growth factors ↑

Matrix Deposition

Production by smooth mucle cells and myofibroblasts ↑
Degradation ↓

Progression of atherosclerosis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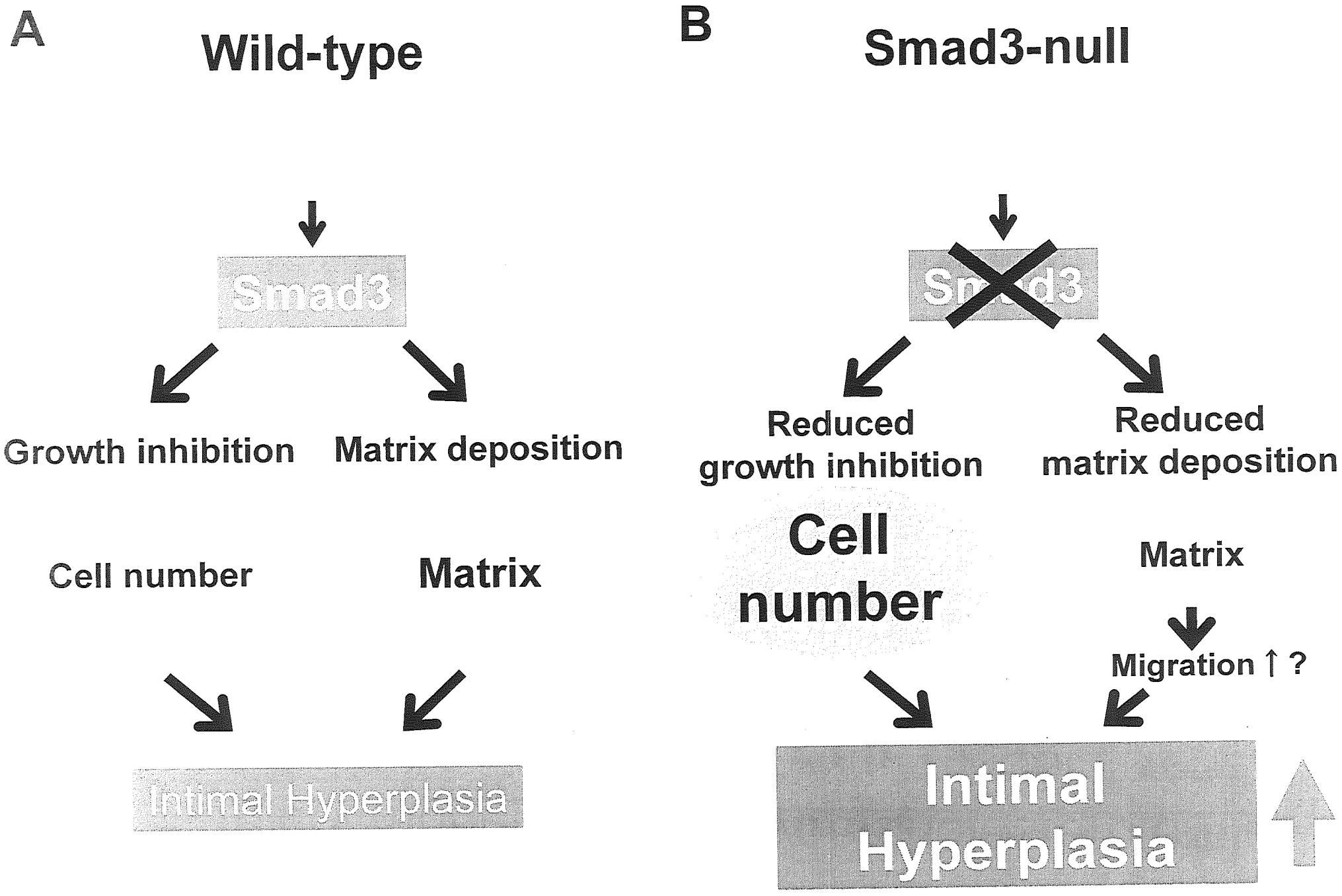
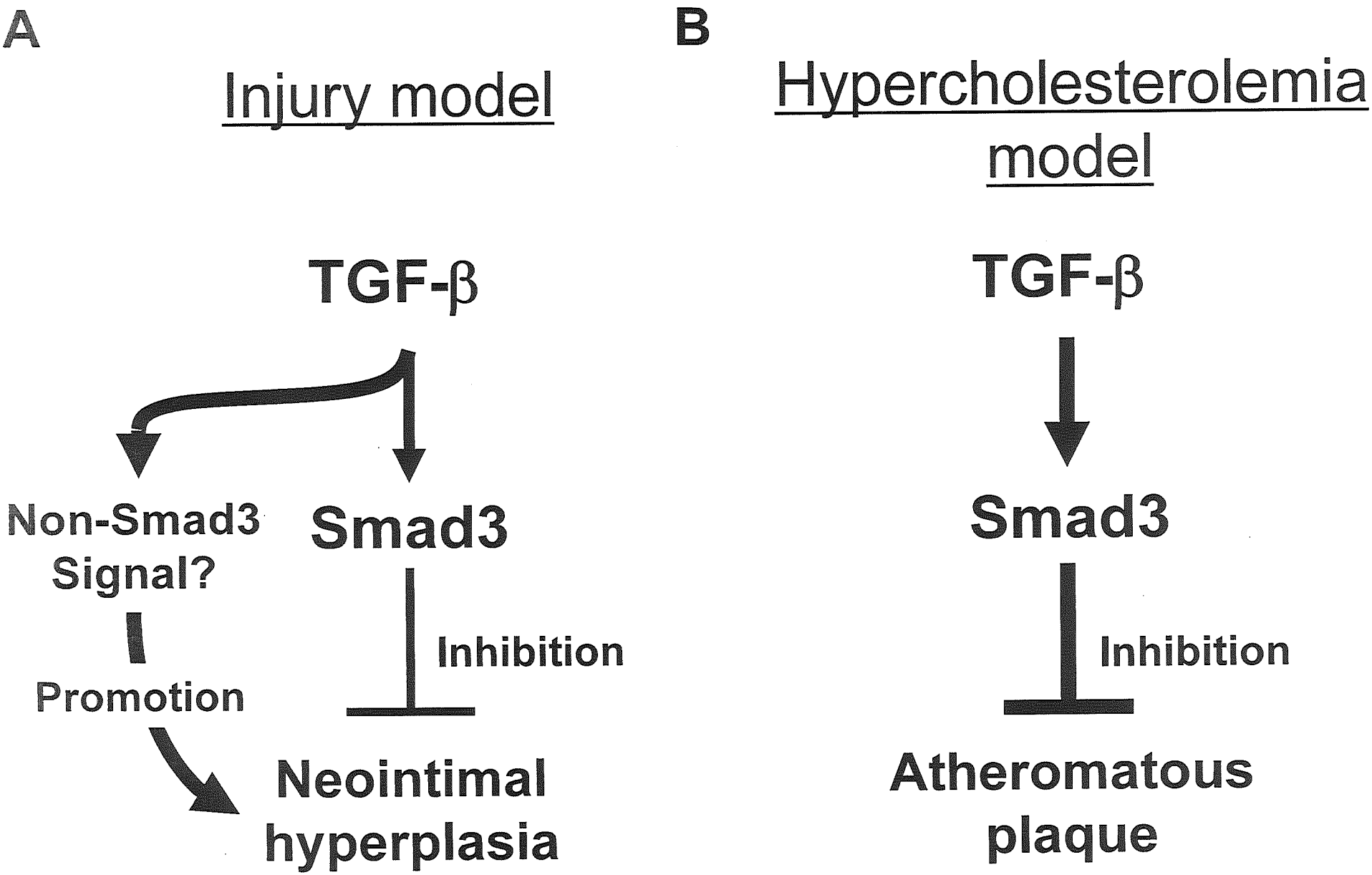

A
Wild-type
Smad3
Growth inhibition
Matrix deposition
Cell number
Matrix
Intimal Hyperplasia
B
Smad3-null
Reduced growth inhibition
Reduced matrix deposition
Cell number
Matrix
Migration ↑ ?
Intimal Hyperplasia


A
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TGF-β
Non-Smad3 Signal?
Smad3
Inhibition
Promotion
Neointimal hyperplasia
B
Hypercholesterolemia model
TGF-β
Smad3
Inhibition
Atheromatous plaque