

From DEPARTMENT OF MOLECULAR MEDICINE AND  
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# NEW PATHOGENIC MECHANISMS IN DIABETIC WOUND HEALING

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Dedicated to all the **MICE** I have used for my experiments



## ABSTRACT

Diabetic foot ulcers (DFU) represent one of the most feared and invalidating complication of diabetes with high financial pressure for the healthcare system. For the moment there is no specific therapy available and it has become a priority to develop novel rational therapeutic strategies based on new pathophysiological mechanisms. Our focus was therefore to delineate relevant pathogenic pathways specifically deregulated in diabetes that could contribute to the defective wound healing in diabetes. Cellular proliferation, migration and differentiation, angiogenesis, extracellular matrix deposition, local recruitment of endothelial precursors cells are some of the essential processes activated during wound healing. We decided to focus our investigation on two central signaling pathways (HIF-1 pathway and Notch signaling) that modulates most of the above cellular events.

Hypoxia plays an important role in the development of DFU. We showed that hyperglycemia complexly repressed the function of Hypoxia inducible Factor (HIF) which is the main cellular adaptor to low oxygen tension. The repressive effect of hyperglycemia on HIF-1  $\alpha$  was pVHL dependent and affected complexly its transactivation. This was mirrored by suppression of several HIF-1 target genes essential for wound healing. However, by blocking HIF-1 $\alpha$  degradation through chemical interference with HIF hydroxylases (DMOG or DFX), it was possible to reverse the repressive effect of hyperglycemia on HIF and to improve the wound healing process in a diabetic mouse model (the db/db mouse). Moreover, local adenovirus-mediated transfer of two stable HIF constructs demonstrated that stabilization of HIF-1 $\alpha$  is necessary and sufficient for promoting wound healing in a diabetic environment. Hyperbaric oxygen therapy (HBOT) has been used as therapeutical option for severe foot ulcers, resistant to standard therapy. The detailed mechanisms activated by HBOT are however still unraveled. We showed that HBOT activated HIF-1 $\alpha$  at several levels with functional consequence on cellular proliferation. Moreover, we could show that local transfer of a stable form of HIF has additive effect to HBOT improving wound healing in the db/db mice.

Notch signaling is a cell-to-cell contact system that consists of several receptors (Notch 1-4) and ligands with a high specific cell-dependent effect. Binding of the ligands to the receptors is followed by proteolytic cleavage of the receptor by a  $\gamma$ -secretase complex which is followed by activation of the intracellular signaling. Here we show that hyperglycemia activated Notch signaling at several levels both *in vitro* and *in vivo*. The effect of hyperglycemia on Notch signaling is canceled in the presence of  $\gamma$ -secretase inhibitors with positive functional effect both on *in vitro* migration and on *in vitro* angiogenesis assays. Moreover local treatment with  $\gamma$ -secretase inhibitors improved wound healing of db/db mice despite chronic hyperglycemia. The effect is specific for diabetes since neither  $\gamma$ -secretase inhibitors nor immunization with a DNA vaccine against Dll4 influenced the wound healing in non-diabetic animals. Using a loss of function genetic approach (specific siRNA and cre/lox system) we showed that Notch 1 has a central pathogenic role in Notch dependent repression of wound healing in diabetes.

In conclusion, we identified two new pathogenic mechanisms important for impaired wound healing in diabetes. Our findings warrant development of specific therapeutics that address HIF and Notch signaling for normal healing of diabetic wounds.

## LIST OF PUBLICATIONS

- I. Botusan IR\*, **Sunkari VG\***, Savu O, Catrina AI, Grünler J, Lindberg S, Pereira T, Ylä-Herttuala S, Poellinger L, Brismar K, Catrina SB.

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\*These authors contributed equally

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Overactive Notch-1 signaling has repressive effects on wound healing in diabetic mice

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- III. Haller BK, Bråve A, Wallgard E, Roswall P, **Sunkari VG**, Mattson U, Hallengård D, Catrina SB, Hellström M, Pietras K.

Therapeutic efficacy of a DNA vaccine targeting the endothelial tip cell antigen delta-like ligand 4 in mammary carcinoma.

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- IV. **Sunkari VG**, Lind F, Botusan IR, Abad K, Liu ZJ, Brismar K, Velazquez OC, Catrina SB.

Hyperbaric Oxygen Therapy (HBOT) activated Hypoxia inducible factor 1 (HIF-1) that contributes to an improved wound healing in diabetic mice.

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3. Ansurudeen, I., **Sunkari, V. G.**, Grunler, J., Peters, V., Schmitt, C. P., Catrina, S. B., Brismar, K., and Forsberg, E. A. (2012) Carnosine enhances diabetic wound healing in the db/db mouse model of type 2 diabetes. *Amino acids* **43**, 127-134
4. Catrina, S. B., Botusan, I. R., and **Sunkari, V. G.** (2010) Hyperglycemia and hypoxia inducible factor, a multifaceted story. *Cell cycle* **9**, 1856
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## LIST OF SOME IMPORTANT ABBREVIATIONS

ARD	Ankyrin repeat domain
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
CTAD	C-terminal transactivation domain
DFU	Diabetic foot ulcers
EPO	Erythropoietin
EPAS	Endothelial PAS domain protein
IPAS	Inhibitory domain PAS protein
MAML1	Mastermind-like protein 1
LNRs	Lin12/Notch repeats
NICD	Notch intracellular domain
NTAD	N-terminal transactivation domain
NLSs	Nuclear localization sequences
ODD	Oxygen-dependent degradation domain
OG	Oxoglutarate
PEST	Proline-glutamate-serine-threonine-rich
PHDs	Prolyl hydroxylase domain proteins
pVHL	Von Hippel–Lindau tumor suppressor protein
HBOT	Hyperbaric oxygen therapy
Hes	Hairy/enhancer-of-split
Hey	Hairy/enhancer-of-split related with YRPW motif
HG	High glucose or hyperglycemia
HIF	Hypoxia-inducible factor
HRE	Hypoxia responsive element
RAM	RBP-J $\kappa$ -associated molecule
RBP-J	Recombination signal sequence binding protein-Jkappa
ROS	Reactive oxygen species
TAD	Transactivation domain
VEGF	Vascular Endothelial growth factor

# **1 INTRODUCTION**

## **1.1 DIABETES**

Diabetes mellitus (DM) is dramatically increasing worldwide. It is estimated that nearly 250 million people are currently affected by DM worldwide and with an expected increase to 400 million in the near future according to the data from International Diabetes Federation (IDF) and the World Health Organization (WHO)<sup>1</sup>. The life time expectancy of patients with diabetes is on average 10% shorter than in non-diabetics individuals due to complications as the disease progresses<sup>2</sup>. An important complication in term of morbidity, mortality and financial costs is diabetic foot ulcers (DFU)<sup>3</sup>.

## **1.2 EPIDEMIOLOGY**

Diabetic foot ulceration represents a major medical, social and economic problem and is coupled with a high rate of mortality<sup>3,4</sup>. Amputation rate in diabetes is almost 15 times higher when compared to the non-diabetic population<sup>5</sup>. About 85% of non-traumatic amputations originate from DFU which is also the most common cause of hospitalization of diabetic patients<sup>6</sup>. DFU has also a high risk for recurrence. The annual incidence of diabetic foot ulcers is between 4 to 10% in diabetic population<sup>7-9</sup>. Almost 25% of patients are at risk to develop foot ulcers during their life time<sup>9</sup>. The therapeutic options available nowadays are restricted to off-loading, treatment of infection and improvement of blood circulation. However even with the best clinical care, the time to heal is longer than 3 months and there are up to 10% of the patients who eventually undergo amputation<sup>4,10</sup>.

## **1.3 ETIOLOGY**

The etiology of diabetic foot ulcers is multifactorial. Diabetic neuropathy and ischemia are central contributors to the development of DFU. Impaired blood flow due to micro and macro angiopathy leads to impaired tissue nutrition that makes the skin more susceptible to trauma.

Neuropathy plays an essential role in the development of DFU. Most of the plantar ulcerations are preceded by distal symmetrical polyneuropathy<sup>11,12</sup>. Motor neuropathy

affects the small muscles of the foot and causes weakness, atrophy, and deformity that create areas exposed to high pressure. Reduction in sweating as a consequence of autonomic neuropathy eventually results in drying and fissuring of the skin and consequent ulceration<sup>13</sup>. Autonomic neuropathy is also the most common predictor of DFU<sup>14</sup>. Moreover, peripheral autonomic neuropathy and hyperglycemia lead to impaired skin microcirculation<sup>15,16</sup>. Sensory neuropathy is an important predisposing factor because it leaves the patients to be exposed to trauma without protective reaction of avoidance. Improper footwear, puncture wounds and foreign bodies in footwear undetected because of lack of sensation result in increased pressure and may lead to ulceration<sup>17-19</sup>. Substantial evidence shows that repetitive pressure result in tissue breakdown and poor healing<sup>20-23</sup>. Callus formation is often seen in patients with diabetic foot exposed to repeated pressure and increase significantly the risk of developing DFU on the same area<sup>24</sup>.

Arterial insufficiency is also important in the defective wound healing in DFU due to the reduction of oxygen supply and nutrition essential for healing<sup>25</sup>. About 46% of amputations in patients with diabetic foot ulcers are due to arterial insufficiency<sup>26,27</sup>.

Infection in the DFU contributes also negatively to the regenerative capacity of the tissues. Two thirds of patients with diabetic foot ulcers are affected by osteomyelitis with its specific therapeutic challenges<sup>28</sup>.

## **1.4 THE WOUND HEALING PROCESS IN DIABETES**

The wound healing process consists of several phases: inflammation, proliferation, granulation and tissue remodeling<sup>29</sup>. Wound healing represent a cellular response to injury and involves activation of several cells i.e. fibroblasts, endothelial cells, macrophages, and platelets. Several growth factors and cytokines are released in a perfectly coordinated manner during the healing process<sup>30</sup>. The first step in wound healing after injury is the clot formation, initiated by release of several factors such as TGF beta and PDGF-B which help in recruiting fibroblasts and leukocytes to the wound area<sup>31</sup>. The Inflammatory phase lasts around 4 days and macrophages play a vital role in this phase. Recently a central role in the inflammatory phase was identified for the plasma protein plasminogen<sup>32</sup>. Macrophages migrate to the wound

area and engulf necrotic material and then produce factors that induce angiogenesis by endothelial cells, epithelialization by keratinocytes and matrix deposition by fibroblasts producing collagen, a major component of extra cellular matrix (ECM)<sup>33,34</sup>. Local endothelial cells migrate to form capillary sprouts<sup>35</sup>. Endothelial progenitor cells derived from bone marrow, home at the site of injury, undergo in situ differentiation and contribute to vasculogenesis<sup>36</sup>. Granulation, re-epithelisation and wound contraction are also important processes of wound healing<sup>37</sup>. Granulation tissue is the fibrous connective tissue, composed of fibroblasts, that typically grows from the base of the wound and they mainly secrete type III collagen<sup>38</sup>. Granulation is followed by epithelisation; during this process epithelial cells and keratinocytes migrate across the wound barrier and granulation tissue. Epithelial cells proliferate and form a sheet across the edges of the wound<sup>38</sup>.

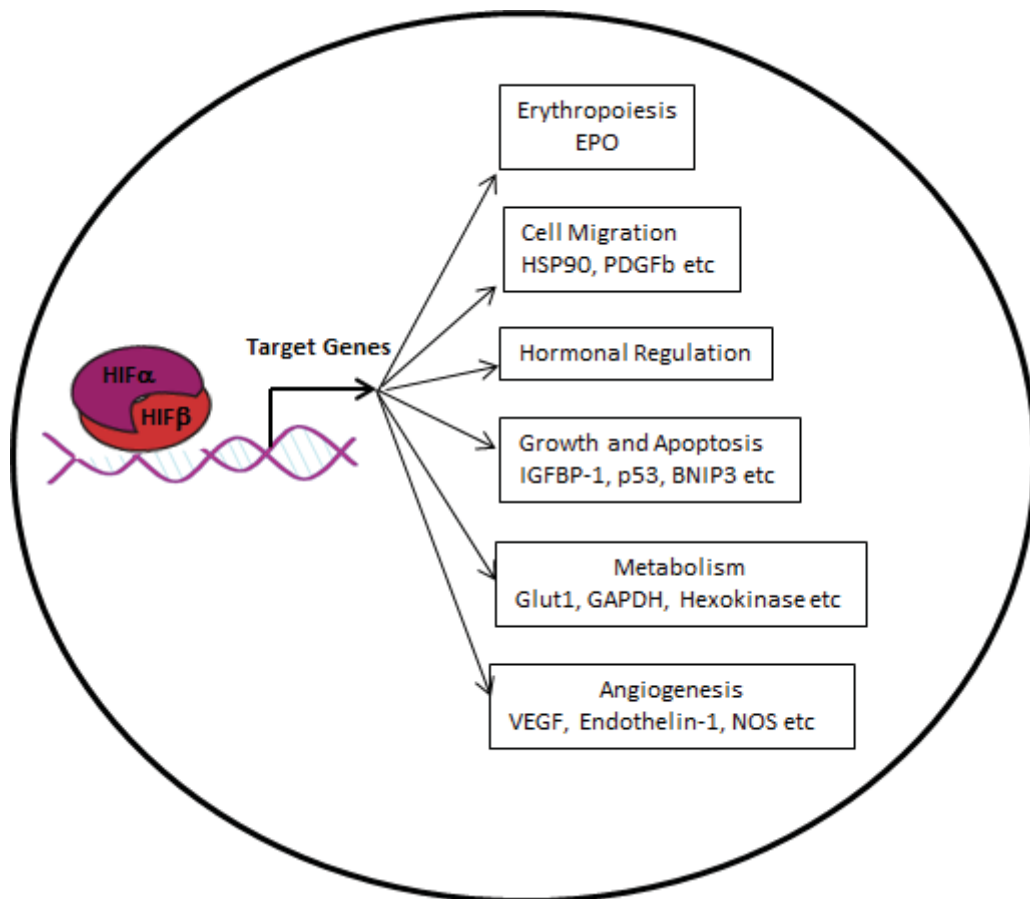
Contraction is also an important step in wound healing<sup>39</sup> where myofibroblasts and fibroblasts are vital contributors. They are stimulated by growth factors and are attracted by fibronectin to move along the fibrin on extracellular matrix to wounds edges. Successful contraction eventually signals for tissue remodeling which is the last phase of wound healing where type 1 collagen has an important role in organizing the collagen fibers in a process that may last from several days to months<sup>40</sup>.

Diabetes alters several mechanisms at cellular and molecular levels that lead to impaired wound healing process<sup>30,41</sup>. Hyperglycemia leads to impairment in migration<sup>42</sup> and angiogenesis<sup>43,44</sup>, dysfunction of macrophages<sup>45</sup>, imbalance in ECM components and their remodeling by MMPs<sup>46</sup>, impaired growth factor production<sup>44,47,48</sup> etc. Markers for delayed wound healing such as c-myc and beta-catenin that have repressive effects on migration of keratinocytes were suggested to be used in order to identify chronic wounds with low potential to spontaneous healing<sup>49</sup>. Endothelial progenitor cells (EPC) replace lost cells at the site of tissue injury. EPCs are critical in neovascularization and play a central role in wound healing, but their circulating levels and their number at the site of the wounds are decreased in diabetes and contribute to a delay in wound healing<sup>50</sup>.

## 1.5 HIF SIGNALING PATHWAY

### 1.5.1 Hypoxia

Hypoxia, defined as a lower oxygen concentration, can be generalized or localized and it is the consequence of either inadequate oxygen supply or increased oxygen consumption. Hypoxia plays an important role in several essential processes such as angiogenesis by triggering vessel growth and promoting vascular bed expansion<sup>51</sup> and playing roles in cell fate decision<sup>52,53</sup>. The molecular reaction to hypoxia is mediated by a transcriptional factor called hypoxia-inducible factor (HIF). HIF activates approximately 100 target genes, which play central physiological roles in response to hypoxia (figure 1)<sup>54,55</sup>



**Figure1: HIF-1 activates several genes with central roles in the reaction to hypoxia**

### 1.5.2 Hypoxia Inducible factors

HIF is a heterodimeric transcription factor first discovered as a regulator of erythropoietin production<sup>56</sup>. The heterodimeric complex is composed of two constitutively expressed subunits: an alpha subunit regulated by oxygen and a beta subunit also called aryl receptor nuclear translocator (ARNT)<sup>57</sup>. HIF belongs to the basic helix-loop-helix (bHLH)-Per-ARNT-Sim (PAS) protein family<sup>58</sup>. The HIF-1 alpha subunit is composed of two transactivation domains, namely NTAD (N-terminal transactivation domain) and CTAD (C-terminal transactivation domain). HIF-1 alpha stability is negatively regulated in normoxia at the oxygen dependent degradation domain (ODD) which is found in NTAD<sup>59</sup>. The N-terminal bHLH is rich in residues that involved in DNA binding<sup>60-62</sup>. Co-factors such as CBP/p300 interact with both HIF-1 alpha transactivation domains to activate gene transcription<sup>63-65</sup>.

Three isoforms of HIF – alpha subunits have been identified (HIF-1 alpha, HIF-2 alpha/EPAS1, HIF-3 alpha) and three HIF- beta subunits (ARNT, ARNT2 , ARNT3) (figure 2). The overall similarity between HIF-1 and HIF-2 is about 48% in their amino acid composition. HIF-1 alpha contains 826 amino acids while HIF-2 alpha has 870 amino acids with longer N-terminal region than HIF-1 alpha<sup>66,67</sup>. The transactivation domains at the C-terminal region have the highest similarity between the isoforms<sup>68</sup>.

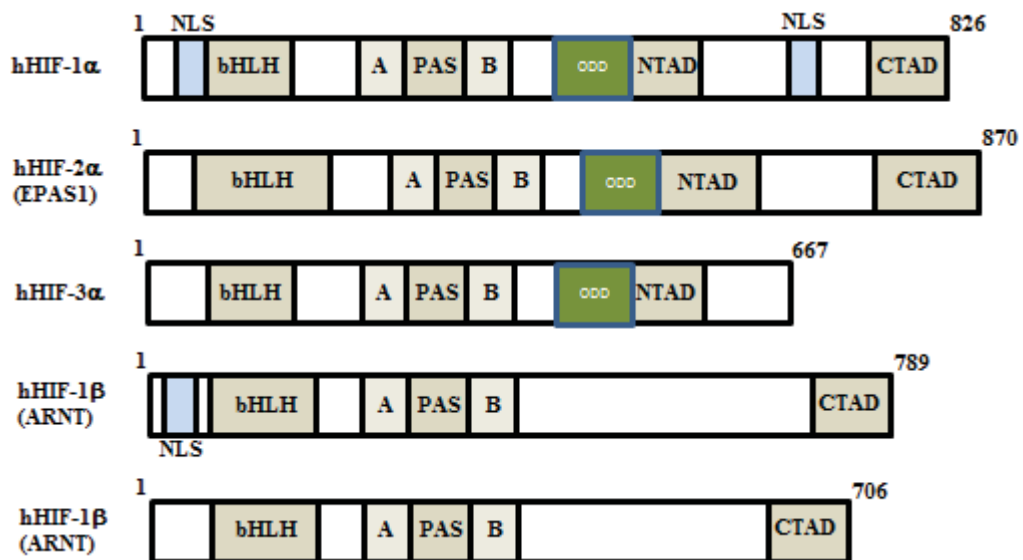


Figure 2: HIF-alpha Isoforms

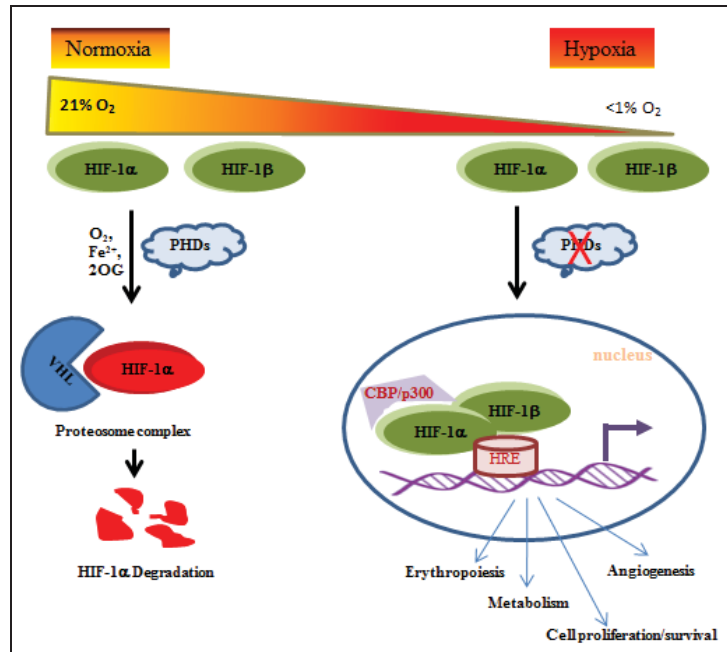
HIF-3 alpha has a different overall structure but with similar N-terminal region that permits dimerization with HIF-1 beta which is followed by binding to the hypoxia responsive element (HRE). Unlike HIF-1/2, HIF-3 alpha lacks CTAD region and is regarded as a dominant negative regulator of HIF signaling<sup>69,70</sup>. Availability of ARNT is crucial for HIF-3 alpha actions<sup>71</sup>. HIF-3 alpha has been identified as a HIF-1 target gene<sup>72</sup> and is also uniquely regulated at the transcription level since HIF-3 mRNA increases under hypoxic conditions<sup>73</sup>.

The expression pattern of HIF-1 alpha and ARNT is ubiquitous, while the other members have a restricted pattern of expression<sup>74</sup>. HIF-2 alpha is expressed in endothelial cells, hepatocytes, cardiomyocytes<sup>66,75</sup>, while HIF-3 alpha is expressed in heart, brain, lung, kidney and adult thymus<sup>76</sup>.

### **1.5.3 Regulation of HIF-1 protein stability**

HIF-1 beta (ARNT) is constitutively expressed irrespective of oxygen levels<sup>77,78</sup> but only HIF-1 alpha is degraded in normoxia via proteasomal degradation (figure 3). In the presence of oxygen, HIF-1 alpha is hydroxylated on at least one of the two conserved proline residues within the ODD (oxygen degradation domain) by prolyl hydroxylases (PHDs)<sup>79-82</sup>. The hydroxylated HIF-1 alpha is identified by von Hippel-Lindau tumor suppressor protein (pVHL) and eventually ubiquitinated leading to its degradation by the 26S proteasome<sup>83,84</sup>. These proline residues which are sensitive to oxygen are conserved in all three isoforms of HIF alpha subunits, HIF-1 alpha (402 and 564), the proline residues in HIF-2 alpha and HIF-3 alpha (405 and 530). Three isoforms of PHDs have been identified (PHD1, PHD2 and PHD3) and belong to the dioxygenase super family, requiring together with oxygen two cofactors (2-oxoglutarate and iron) for performing its activity<sup>82,85-88</sup>. PHD2 seems to be the most important PHD2 for regulation of HIF stability in normoxia<sup>89</sup>, but the expression pattern and distribution of PHDs members are tissue and cell specific<sup>90-92</sup>.





**Figure 3: Classical HIF-1 alpha regulation.**

Interestingly PHDs are induced by HIF-1 in prolonged hypoxia closing a regulatory feed-back loop<sup>93</sup>.

HIF-1 alpha is also regulated by other factors besides the canonical regulation through PHDs. Acetylation of Lys 532 by Arrest defective protein-1 (ARD1) that is modulated by oxygen, enhances interaction of HIF-1 $\alpha$  with pVHL and subsequently leads to HIF-1 $\alpha$  degradation<sup>94</sup>. However, metastasis-associated protein 1 (MTA1) counteracts the activity of ARD1 by binding to HIF-1 $\alpha$  and enhancing the stability of HIF-1 alpha<sup>95</sup>. Protein SSAT2 promotes ubiquitination of HIF-1 alpha by interacting with HIF-1 alpha, pVHL and elongin C<sup>96</sup>. Osteo Sarcoma protein-9 (OS9) initiates a strong binding between HIF-1 and PHD2 ensuring a stable complex formation and enabling a perfect degradation pathway<sup>97</sup>. The affinity of pVHL to HIF-1 alpha binding is decreased by Hepatitis B virus X protein (HBx), a protein involved in hepatocellular carcinoma development, interacts with the bHLH/PAS domain of HIF-1 and decreases its binding to pVHL, thereby increasing stability of HIF-1 alpha<sup>98</sup>. Bafilomycin stimulation allows ATP6V0C interaction with HIF-1 alpha and directly competes with pVHL in binding to HIF-1 alpha<sup>99</sup>. The pVHL-interacting deubiquitinating enzyme 2 (VDU2) interacts with the HIF-1 alpha and pVHL complex and reverses the ubiquitination process and stabilizes HIF-1 alpha<sup>100</sup>. Insulin like growth factor -1 (IGF-1) induced accumulation of both HIF-1 alpha and HIF-2 and followed by an increase in HIF function<sup>101,102</sup>.

SIRT3, one of the proteins from the Sirtuin family destabilizes HIF-1 alpha in a PHD dependent manner. It has been shown that SIRT2 destabilizes HIF-1 alpha by inhibiting ROS production, promoting secondary maximal PHD activity<sup>103</sup>. Reactive oxygen species (ROS) play a key role in HIF-1 alpha stability; it has been shown that ROS directly affects PHDs enzymatic activity by preventing hydroxylation of HIF<sup>104</sup>; ROS generated from the mitochondrial complex were suggested to play a special role in HIF stabilization<sup>105</sup>. However, the specific role and relative significance of ROS in mediating the hypoxic response remain unclear and contradictory, as lower levels of ROS were detected in hypoxia than in normoxia<sup>106</sup>.

HIF-1 alpha is also regulated independently of the pVHL mechanism through several interacting proteins. Geldanamycin which is an HSP90 antagonist promotes ubiquitination and proteasomal degradation of HIF-1 $\alpha$  by disrupting its association with HSP90, in renal carcinoma cell lines via a PHD2/pVHL-independent pathway<sup>107</sup>. RACK1 binds to HIF-1 alpha and promotes its degradation independent of pVHL<sup>108</sup>. This process can be enhanced by SSAT1 (Spermidine/spermine N(1)-acetyltransferase 1) stabilizing interaction of HIF-1 alpha to RACK1<sup>109</sup>. RACK1 competes with HSP90 in binding to HIF-1 alpha thereby promoting a VHL independent degradation<sup>108</sup>. Binding of p53 to a HIF and p300 transcriptional complex mediates inhibition of HIF activity<sup>110</sup>, HIF-1 $\alpha$ -p53 interaction is modulated by several proteins such as Mdm2 (murine double minute, functions as E3-ubiquitin ligase and negatively regulate p53 actions)<sup>111</sup> or Jun activation domain-binding protein-1 (Jab1), a coactivator of AP-1 transcription factor interacts directly with HIF-1 alpha and increased transcription and stability of HIF-1 alpha protein, through modulation of HIF-1 $\alpha$ -p53 interaction<sup>112,113</sup>.

Small ubiquitin-related modifier (SUMO) modulates also HIF-1 alpha stability. It has been shown that hypoxia induces SUMOylation of HIF-1 by binding it to the pVHL-E3 complex which leads to HIF-1 to degradation<sup>114</sup>. On the other hand RSUME which is an inducer of SUMOylation increases SUMO conjugation with HIF-1 alpha in hypoxia and stabilizes HIF-1 alpha<sup>115</sup>. The data available to date about SUMO and HIF-1 alpha regulation are confusing and further research is needed to understand the exact role of SUMOylation.

#### 1.5.4 Regulation of HIF-1 alpha transcriptional activity

The transcriptional activity of HIF-1 alpha is modified and regulated by many interacting proteins; HIF-1 alpha forms a heterodimeric complex with ARNT and binds to promoter of hypoxia responsive elements (HREs) in the nucleus to express its downstream target genes<sup>116</sup>. Factor inhibiting HIF (FIH) hydroxylates asparagine-803 (HIF-1) and asparagine-851 (HIF-2) in CTAD in the presence of oxygen and modulates their transcriptional activity. This hydroxylation prevents the binding of co-factors CBP/p300 and thereby represses the transcriptional activity of HIF in normoxia. FIH also requires 2-oxoglutarate and iron for its activity like PHDs being member of the same family of dioxygenase<sup>64</sup>. FIH has a lower  $K_m$  for oxygen than PHDs have, making the PHD respond to a lesser decrease in oxygen levels<sup>117</sup> and become inactive to stabilize HIF-1 alpha, while at the same time transcriptional activity of HIF is still be inhibited by FIH experiencing saturating oxygen concentrations.

Other PAS family members such as MOP3 and MOP9 interact with HIF-1 alpha to form active transcription heterodimers; however their targets are still elusive<sup>118-120</sup>. P14<sup>ARF</sup> tumor suppressor protein sequesters HIF-1alpha to nucleolus and inhibits its transcriptional activity<sup>121</sup>. PER2 (Period circadian protein homolog 2), a circadian factor prevents the transcriptional activity of HIF-1 and ARNT complex, possibly through a protein-protein interaction<sup>122</sup>. Contrarily, proteolytical degradation of PER1 is prevented by interaction of HIF-1 $\alpha$  with PER1<sup>123</sup>. COMMD1 (COMM domain-containing 1) protein binds to the amino terminus of HIF-1 alpha and prevents the dimerization of HIF-1 $\alpha$  with HIF-1 $\beta$  thereby modulating HIF-1 alpha degradation as well<sup>124</sup>.

HIF $\alpha$  mRNA stability is also crucial for HIF $\alpha$  protein levels and function. HIF mRNA can be negatively regulated by mRNA-destabilizing protein TTP (tristetraprolin) that directly binds to 3'UTR of HIF-1 mRNA and regulates HIF-1 alpha levels in hypoxia<sup>125</sup>. MiR155 represses HIF-1 alpha mRNA, protein and its transcriptional response during hypoxia<sup>126</sup>. It is interesting to note that miR155 is induced by HIF-1 during prolonged hypoxia resulting in a negative feedback loop mechanism. Also miR17-92, directly represses HIF-1 interestingly just in normoxia but not in hypoxia<sup>126,127</sup>.

Co-activators play a vital role in initiation of HIF-1 alpha transcriptional activity. PKM2 enhances binding of HIF-1 alpha to HREs to promote transcription under hypoxic conditions. PKM2 is also induced by HIF-1 resulting in a positive feedback loop mechanism<sup>128</sup>. SUMO1/sentrin/SMT3 specific peptidase-3 SENP3 enhances binding of p300 to HIF-1 alpha and increases transcription by de-SUMOylation of p300 during mild oxidative stress<sup>129</sup>. Pontin, a chromatin remodeling factor is a coactivator of HIF-1 alpha by mediating the interaction of HIF-1 and p300 for initiating transcription in hypoxia<sup>130</sup>. A growth suppressor, Necdin, inhibits transcriptional activity of HIF-1 alpha during hypoxia by association with ODD domain. Moreover Necdin decreases HIF-1alpha protein level and mediates HIF-1 degradation<sup>131</sup>. SIRT1 deacetylates lysine-674 on HIF-1 alpha and impedes p300 recruitment and transactivation of target genes while p300/CBP-associated factor (PCAF) acetylates the same residue and enhances binding of p300 with HIF1 $\alpha$  for initiating transcription<sup>132</sup>. It has been shown that CITED2 and CITED4 bind to p300/CBP preventing its interaction with HIF-1 alpha thereby interfering with HIF-1 alpha activity<sup>133,134</sup>. Estrogen-related receptors (ERRs) serve as cofactor for HIF-1 alpha during hypoxia, by direct interaction<sup>135</sup>.

Several other interacting proteins are involved in the recruitment of coactivators and modulate HIF transcription. For example, CTAD of HIF-1 alpha is modified by a thiol-redox regulator Ref-1, which ultimately facilitates the binding of coactivators<sup>136,137</sup>. Histone deacetylase 7 (HDAC7) forms a complex with HIF-1 $\alpha$  and p300 under hypoxic conditions and increases HIF-1 $\alpha$  transcriptional activity<sup>138</sup>. Phosphorylation of HIF-1 alpha by MAPK enhances the transcriptional activity without affecting HIF-1 alpha stability or DNA binding capacity<sup>139</sup>.

### **1.5.5 HIF function**

When the oxygen levels decrease during hypoxia, PHDs activity is inhibited leading to the stabilization of HIF-alpha. Stabilized HIF translocates to the nucleus and dimerises with ARNT (HIF-1 beta)<sup>140</sup> and binds to HREs to promote transcription of approximately 100 target genes<sup>54,55,139</sup> that participate in regulation of several processes such as erythropoiesis (erythropoietin), angiogenesis (vascular endothelial growth factor, Angiopoietin 1 and 2, E-cadherin etc), glucose and energy metabolism (glucose

transporter, phosphoglycerate kinase 1), cell differentiation, cell survival, apoptosis, tumor development etc<sup>141-144</sup>.

HIF also plays a pivotal role for development during embryogenesis. HIF-1 alpha knockout mice stop the development at E8.5 and eventually die at E10.5 due to vascular defects and abnormalities in cardiac and neural developments<sup>145-147</sup>. Like HIF-1 alpha knockout mice, HIF-2 alpha knockout mice have high embryonic lethality between E9.5 and E16.5<sup>148-150</sup>. However, replacement of HIF-1 alpha in HIF-2 knock mice cannot rescue them from lethality<sup>151</sup>. This shows the distinct functions of HIF-1 alpha and HIF-2 alpha despite the high level of sequence homology, at least during embryo development. Haploinsufficiency of HIF-2 alpha (EPAS<sup>-/-</sup>) resulted in a strain-specific phenotype in mouse with metabolic defects and impaired homeostasis towards reactive oxygen species (ROS)<sup>150</sup>. HIF-1 beta also plays a vital role in embryo development, as deletion of HIF-1 beta leads to death due to vascular and placental abnormalities<sup>152,153</sup>.

HIF is also involved in many other processes such as migration, proliferation, wound healing, ischemia, inflammation, differentiation etc. Hypoxia through HIF induces migration of fibroblasts and keratinocytes<sup>154,155</sup> and induces members of the integrin superfamily (CD11b/CD18) that enhance the migration of leukocytes<sup>156,157</sup>.

In general the undifferentiated state of cells is maintained in hypoxia. However the effect is cell specific as hypoxia inhibits differentiation of preadipocytes and myoblasts<sup>158,159</sup> but promotes differentiation of other cell types, such as neural crest stem cells and mouse mammary epithelial cells<sup>160,161</sup>. Specific functions of HIF-2 alpha were also identified in controlling the undifferentiated state. Oct-4 which is important in maintaining the undifferentiated state of stem cells is controlled only by HIF-2alpha<sup>162</sup>.

HIF pathway plays an important role in regulating the inflammation response as well. Inactivation of the HIF-1 pathway leads to inhibition of motility, cell aggregation and pathogen killing capacity after phagocytosis of myeloid cells<sup>163</sup>. Hypoxia also promotes the secretion of inflammatory chemokines<sup>164</sup> important to attract monocytes and neutrophils<sup>165</sup>. Interestingly the chemokines activate a positive feedback on HIF signaling mediated by ROS that stabilize HIF-1 alpha<sup>165</sup> and by an additional effect on increasing the DNA binding capacity of HIF-1 alpha and consecutively expression of

HIF-1 target genes<sup>166</sup>. Moreover hypoxia regulates the reaction to inflammation through HIF independent mechanisms as well. NF- $\kappa$ B that has an essential role in inflammatory and immune responses is induced in hypoxia through a direct effect on I $\kappa$ B<sup>167</sup>.

Wound healing occurs in a relatively hypoxic milieu being generally surrounded by damaged vessels with impaired blood flow but having in the same time high oxygen need for sustaining the healing process. Hypoxia promotes the angiogenic process and tissue repair by induction of many target genes important for wound healing<sup>168</sup>. It has been shown that motility of keratinocytes was increased due to hypoxia around the wound, and this promotes wound healing by activating re-epithelialization and closure of wounds<sup>155</sup>. HSP90 alpha for instance which is induced by HIF-1 alpha stimulates migration of fibroblasts and healing<sup>169</sup>.

## **1.6 NOTCH SYSTEM**

The Notch signaling pathway contributes to the multi-cellular development by controlling cell fate decisions and consequently, morphogenesis. Notch signals control cellular lineages by linking the fate of one cell to that of a neighboring cell, through the interaction of the Notch surface receptor expressed on one cell with membrane-bound ligands expressed on the surface of an adjacent cell. The first report on Notch described a novel phenotype in the fruit fly *Drosophila melanogaster*, characterized by a notched wing margin<sup>170-172</sup>. After the initial findings two decades later, in 1940 it has been shown that homozygosity for the notched wing allele resulted in embryonic lethality and the neural tissue expanded at the expense of ectoderm, establishing Notch as a controller of cell fate decisions from uncommitted progenitors. The research on Notch system has expanded enormously after the cloning of the Notch gene establishing the role of Notch signaling in disease and development<sup>173</sup>.

### **1.6.1 A molecular overview of the Notch System**

Notch signaling operates between juxtaposed cells, where membrane bound receptors (signaling receiving cell) and ligands (signal sending cell) need a close physical contact to initiate the signaling process. The Notch receptor in itself is the effector molecule of

the pathway and operates without need of second messengers (such as cGMP, cAMP etc), a common feature for other signaling pathways.

### 1.6.2 Notch Receptors

Notch receptors have developed during evolution from just one Notch receptor in *Drosophila*, 2 Notch receptors in *C. elegans* to reach 4 different Notch receptors (Notch1-4) in most vertebrate species. The Notch receptors, Notch 1-4 (figure 4) are single-pass transmembrane proteins composed of 29-36 tandemly arranged EGF repeats<sup>174-177</sup>. The EGF repeats 11 and 12 play an important role in the interaction of the ligand to the receptor in order to initiate signaling<sup>178</sup>. Moreover the extracellular domain has three cysteine-rich family specific LNR domains (Lin Notch Region present only in Notch related proteins at C-terminal to EGF repeats). LNR region negatively regulates Lin proteins (Lin proteins facilitate the intracellular signaling) and participates in maintaining Notch receptors in resting state before binding to ligand. The Notch intracellular domain (NICD) contains RAM (RBP-Jk associated molecule) domain and several ankyrin repeats that are involved in interaction with other proteins such as the CSL complex<sup>179-181</sup>. Other intracellular domain includes a C-terminal PEST region and RE/AC domain<sup>182</sup> that confers the transactivating capacity and the specificity of the Notch receptor by binding to RBP-Jk<sup>182,183</sup>. The PEST domain is believed to be important for stability and ubiquitination<sup>184,185</sup>. On both sides of the ankyrin repeats region NICD has two nuclear localization signals (NLSs)<sup>186,187</sup>. Notch receptors 1-3 have two NLSs, whereas Notch4 has one NLS with a small intracellular domain<sup>188</sup>.

After translation and insertion into the endoplasmic reticulum membrane, the newly synthesized Notch receptor interacts with O-fucosyltransferase-1(Ofut1), an enzyme catalyzing the addition of fucose sugar moieties to EGF repeats of Notch<sup>189,190</sup>. The Notch receptor is subsequently transported through the secretory pathway to the Golgi network for further glycosylation with importance for future interaction of the receptor with the ligand which is catalyzed by the fringe family of glycosyl transferases<sup>191-193</sup>. Fringe originally identified in *Drosophila* has three mammalian homologs – lunatic, radical and manic fringe<sup>194-196</sup>.

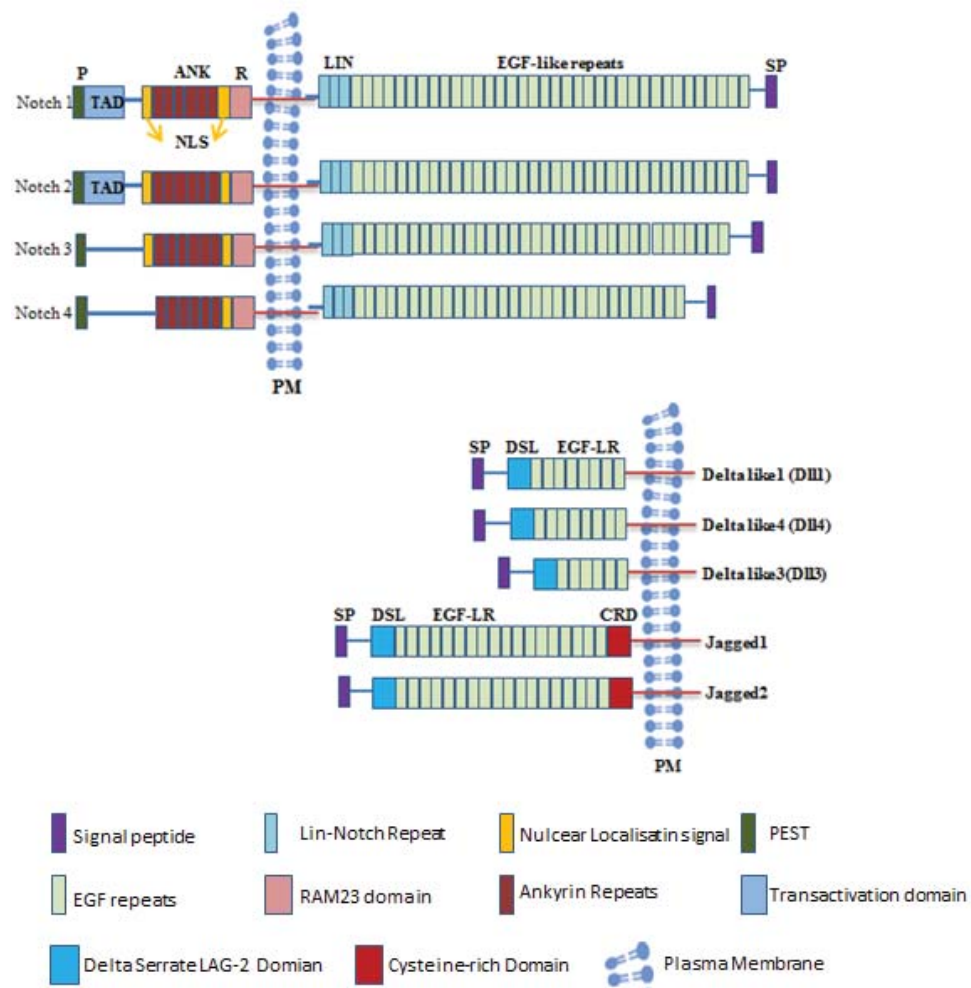


In the trans-Golgi network Notch undergoes a proteolytic processing event (known as site 1 or S1 cleavage) mediated by a furin-like convertase enzyme<sup>197,198</sup>. The cleavage is important for maturation of the protein and for creation of a heterodimeric Notch receptor. Only trace amounts of non-S1 cleaved forms of Notch can be found at the cell surface<sup>199</sup>.

### 1.6.3 Notch ligands

There are at least 5 functional Notch ligands in vertebrates: three orthologs of *Drosophila* Delta (Delta or Delta-like [Dll] 1<sup>200</sup>, 3<sup>201</sup>, and 4<sup>202</sup>) (figure 4) and two of *Drosophila* Serrate (Jagged1<sup>203</sup> and Jagged2<sup>204</sup>). All ligands are able to interact with all the Notch receptors and induce the second cleavage at the extracellular level<sup>205-207</sup>. However, all ligands have different expression patterns and their specific deletion/inhibition results in diverse outcome<sup>208</sup>. Like Notch receptors, Notch ligands are also single-pass transmembrane proteins and are also composed of a large and variable number of EGF-like repeats in their extracellular domain but with a small intracellular portion. Notch ligands internalize into endosomes to achieve Notch activation and present themselves at the cellular membrane in the signal sending cell. This process is regulated by an ubiquitination assisted by E3-ubiquitin ligases Mindbomb and Neuralized<sup>209</sup>. This step is central in Notch signaling as deficiency in Mindbomb leads to defective Notch activation<sup>210,211</sup>. The N-terminal part of Notch ligand has a DSL (Delta and Serrate/jagged in *Drosophila* and vertebrates, Lag2 in *Caenorhabditis elegans*) domain which is important for Notch-Ligand interaction<sup>212,213</sup>. In contrast to Notch receptors, Notch ligands have a relatively short intracellular domain that contribute to endocytosis and interaction with different intracellular proteins<sup>214</sup>. Also the existence of non-DSL ligands have been proposed, including F3/contactin (which acts as a ligand in oligodendrocyte maturation)<sup>215</sup> and DNER (which acts as neuron specific Notch ligand)<sup>216</sup>. However more studies are needed to understand the mechanism behind this ligand-receptor interaction.

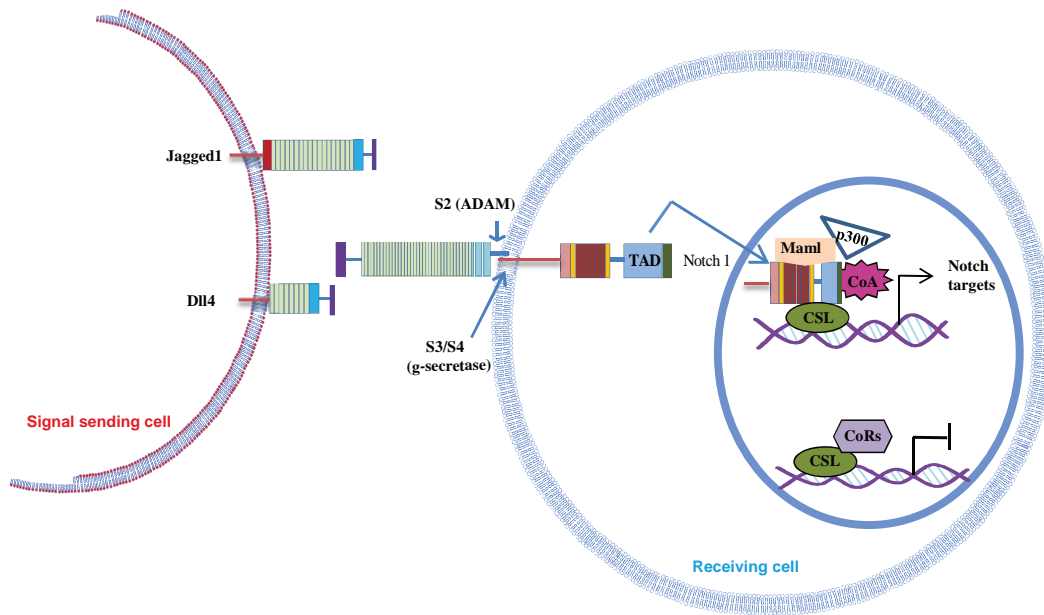




**Figure 4: Mammalian Notch Receptors and Ligands**

#### 1.6.4 Notch Activation

The key to Notch receptor activation is the regulation of ectodomain shedding. The interaction of Notch Ligands (signal sending cell) with Notch receptors (signal receiving cell) elicits two essential proteolytic cleavages (figure 5). First the binding of ligand to receptor induces a conformational change in LNRs present in the negative regulatory region of Notch receptors, which is followed by exposure of S2-site open for cleavage and leads the DSL sites of the ligand for endocytosis<sup>217-220</sup>. An S2 cleavage is dependent on ligand binding and is done by the metalloprotease ADAM called TACE (TNF- alpha converting enzyme)<sup>221,222</sup> followed by an S3/S4 intramembraneous cleavage in the hydrophobic milieu of the lipid bilayer by the large gamma-secretase enzyme complex<sup>223-225</sup>.



**Figure 5: Activation of the Notch signaling pathway**

Gamma-secretase cleavage releases the NICD (Notch intracellular domain) into cytoplasm that translocates to the nucleus<sup>226</sup>. NICD binds to CSL (named after CBF1, Su(H) and LAG-1), a DNA binding protein also known as RBP-Jk (recombination signal sequence binding protein-Jkappa) that is ubiquitously expressed (CBF1 in drosophila)<sup>227,228</sup>. NICD subsequently recruits its coactivators, mastermind-like proteins (MAML) and CBP/300 to promote transcription<sup>229-233</sup>. Turnover mechanism is important in controlling Notch signaling. MAML enhances phosphorylation of NICD and the turnover process<sup>229</sup>. NICD in the nucleus is quite unstable<sup>234</sup>. NICD ubiquitination takes place when MAML phosphorylates the PEST domain that leads to proteasomal degradation<sup>231</sup>.

### 1.6.5 Notch downstream target genes

Several target genes are modulated by Notch signaling. Enhancer of split complex in *Drosophila* was the first identified Notch target gene<sup>235,236</sup>. Other classical Notch target genes are Hes<sup>237</sup> gene family (Hairy/Enhancer of split) and Hey<sup>238</sup> genes which are closely related to Hes gene family and are direct downstream targets of Notch signaling. Following the activation of the Notch receptor, NICD is recruited and induce transcription of these genes by binding to CSL on their promoter regions<sup>237,239</sup>. To date

seven Hes (Hes 1-7) and three Hey (Hey 1-3) genes have been identified<sup>240</sup>. It has been shown that Hes1, Hes5, Hes7 and Hey gene family are Notch target genes<sup>241</sup>. Hes and Hey genes encode basic helix-loop-helix transcription factors of the class C type that act as transcriptional repressors<sup>242,243</sup>. The transcriptional repression blocks the expression of several genes i.e. MyoD<sup>181</sup> in developing muscles or neurogenin<sup>244</sup> in nervous tissue that are important in promoting tissue differentiation. Hes and Hey bind to co-factors such as E12/E47 and sequester them from binding partners such as Mash1 and MyoD which are important for inducing a transcriptional response<sup>181,244</sup>. In this way Hes and Hey acts a transcriptional repressor proteins without binding to the DNA promoter sites. Several other genes modulated by Notch signaling were identified through genome-wide transcriptome analysis, i.e CyclinD1 (coordinates mitotic events), c-Myc (cell proliferation and cancer), p21 (regulator of cell cycle progression), NFkB2 (regulates apoptosis), Snail (assists in mesoderm formation), ADAM19 (Involved in cell-matrix interaction, neurogenesis etc.) and Bcl-2 (apoptosis regulator protein)<sup>241,245,246</sup>. Nrarp is activated by a CSL-dependent Notch activation and have a blocking effect on Notch signaling<sup>247</sup>. Deltex1 (controls cell fate of neural progenitor cells by blocking binding partners such as Mash1<sup>248</sup>) is another negative regulator of Notch signaling but also a Notch target gene.

### **1.6.6 Notch function**

As mentioned before, Notch plays an essential role in embryonic development but also in tissue homeostasis during the adult life, by regulating cell fate decisions, proliferation, differentiation and apoptosis<sup>249-251</sup>. Lateral inhibition is an important Notch function, where the signaling restricts cell fate decisions and differentiation for example early differentiating cells signals to the neighboring cells to not differentiate into the same cell type<sup>252</sup>. Functions of Notch signaling are cell-context dependent. In consequence Notch promotes proliferation of leukemia cells by inducing c-Myc<sup>253</sup> but suppress proliferation of lung cancer cells via p21 and p27<sup>254</sup>. The oncogenic role of Notch was first described in T-cell acute lymphoblastic leukemia (T-ALL)<sup>174</sup>. Gain or loss of function of Notch is associated with other cancers such as skin cancers<sup>255</sup>.

Notch plays a key role in Central Nervous System (CNS). Notch signaling inhibits differentiation of uncommitted stem cells and stops their differentiation into neurons<sup>256-</sup>

<sup>258</sup>. Notch receptors are also expressed in post-mitotic neurons and their activation leads to change in neurite morphology (decrease in neurite elongation and increase in their branching<sup>259,260</sup>). Notch controls also other cells types in CNS such as oligodendrocytes by inhibiting their maturation and differentiation into myelinating oligodendrocytes<sup>261</sup>.

Notch signaling is central in early embryogenesis by guiding the somites and giving rise to axial skeleton in vertebrates through Hes transcription factors<sup>262</sup>. Missense mutations in notch signaling are followed by defects in axial skeleton as in Dll3<sup>263</sup> or in glycosyltransferase Lunatic fringe<sup>264</sup>. Muscle development is also modulated by Notch signaling by upregulating Hes and Hey genes, which represses MyoD which actively participate in promoting myogenic differentiation<sup>181</sup>.

The Notch system has a profound effect on the vascular system as well. Notch1 deficiency leads to a phenotype with vascular malformations<sup>265</sup>. Notch1 alone or Notch1 and 4 double knockout mice exhibit a defect in the vascular remodeling, a similar phenotype that was observed in Jagged1 knockout mice<sup>266</sup>. Notch4 homozygous mutants developed however normally and are fertile<sup>265</sup>. Recent studies pointed out the relative importance of Dll4 in vascular development. Even lack of a single Dll4 allele in mice leads to vascular defects and embryonic lethality, exactly like Notch1/Notch4 double knockout mice<sup>267-269</sup>. On the other hand Dll4 is overexpressed in tumor vessels compared to normal adjacent vascular tissues<sup>270,271</sup>. Dll4 activates Notch in neighboring cells and restricts endothelial sprouting and proliferation by suppressing VEGF receptors<sup>272,273</sup>.

Recent advances suggest a role of Notch signaling in diabetes as well. Pharmacological blockade of Notch signaling with  $\gamma$ -secretase inhibitors or haploinsufficiency of Notch1 markedly increases insulin resistance via FoxO1 dependent manner<sup>274</sup>. Moreover, Notch signaling activation was observed in diabetic nephropathy<sup>275</sup> with unclear specificity since stimulated Notch signaling is present in most of the glomerulosclerotic diseases<sup>276</sup>.

## 1.7 DNA VACCINES

DNA vaccines are made from DNA sequence coding for the antigenic protein of interest which is inserted into a plasmid vector. Inserted DNA is then translated in the host with production of the antigenic protein that will elicit an immune response<sup>277,278</sup>.

DNA vaccines activate the cell-mediated immunity together with humoral immunity<sup>279-281</sup>. DNA vaccination is safer (no risk of infection), cheaper (no need of costly equipment for synthesis), easier to store (can be stored in ambient temperatures) and use, compared with the conventional vaccination methods. Traditional vaccines can be potentially fatal since weakened form of an infectious organism is delivered into the host. They provide primarily just humoral immunity and refrigeration is needed for storage<sup>282</sup>. There has been an explosion of patents applied for DNA vaccines in the last decade and several DNA vaccines are already in clinical trials for different diseases like influenza, malaria, hepatitis, HIV and tuberculosis<sup>277</sup>.

In 1990 it was first shown a sustained expression of a protein in the mouse quadriceps by injecting DNA encoding a lactase driven reporter genes<sup>283</sup>. Subsequent studies showed that DNA delivery (vaccination) has a high potential and can overcome the problems posed by protein and carbohydrate vaccines. Injection of a plasmid containing DNA coding for the influenza nuclear viral protein (antigen) into mouse muscle results in increased CD8<sup>+</sup> cytotoxic T lymphocytes and protecting the mice from recurrent influenza challenges<sup>284</sup>.

DNA vaccines can be delivered either intramuscularly, intradermally or intraperitoneally. Several delivery techniques have been employed as electroporation and gene gun techniques<sup>277</sup>. In recent years Nano particles<sup>285</sup> were used to both protect DNA from degradation and to increase the phagocytic activity of APCs (antigen presenting cells).

## 1.8 HYPERBARIC OXYGEN THERAPY

Hyperbaric oxygen therapy (HBOT) is exposure to pure oxygen (100%) at higher pressure (>1 bar) than at the sea level. HBOT has been used for many years as a therapeutic agent for several indications such as carbon monoxide and cyanide poisoning<sup>286,287</sup>. HBOT has also been used as an adjuvant treatment for patients with diabetic foot ulcers<sup>288</sup>.

HBOT activates several mechanisms with potential relevance for improving wound healing. HBOT improves neutrophil function and helps to combat bacterial infections and can be used as an alternative therapy for treating drug resistant microorganisms<sup>289-292</sup>. HBOT has a vasoconstrictory effect having a positive effect by reducing edema around the wounded tissues<sup>287</sup>. HBOT stimulates the activity of osteoclasts, activates fibroblasts and stimulates angiogenesis with potential positive effects on wound healing<sup>293</sup>.

Angiogenesis (formation of new blood vessels from pre-existing vessels) and vasculogenesis (formation of new vessels by endothelial progenitor cells) are two main mechanisms that contribute to neovascularization. Angiogenesis is stimulated by local factors such as VEGFA and vasculogenesis by recruitment and differentiation of endothelial progenitor cells (EPC)<sup>294-296</sup>. Extracellular matrix (ECM) is the largest component of skin and is composed of polysaccharides, collagen proteins etc. HBOT enhances extracellular matrix formation, an oxygen dependent process important for neovascularization and wound healing<sup>297-300</sup>. Stem/progenitor cells migrate to the site of tissue injury, replace the cells lost and act as a repair system for the wound healing process. Nitric oxide synthase 3 (NOS-3) activity is important for EPC mobilization<sup>301</sup> and exposure to HBOT rapidly increased the EPC mobilization by increasing the NO synthesis in humans and mice<sup>302</sup>. However EPC mobilization is impaired in diabetic patients probably due to decreased NOS-3 activity in presence of high glucose and insulin resistance<sup>302-304</sup>. In reperfusion studies it has been shown that leukocytes bind to ischemic tissues, releases free radicals and proteases leading to damage of the tissues<sup>305,306</sup>. Hyperbaric oxygen treatment reduces the adherence of leukocytes and improves the recovering of the ischemic tissues<sup>307</sup>. Moreover HBOT has several anti-infectious effects as it improves bactericidal action of leukocytes<sup>308</sup>, increases free radical production and oxidation of proteins and lipids in the bacterial membrane

inhibiting consequently the bacterial metabolic function<sup>309,310</sup>. Also, an increase in partial oxygen pressure in the environment creates an unfavorable condition for anaerobic bacteria<sup>311</sup>.

HBOT has potential complications although with low incidence as middle ear barotraumas<sup>312,313</sup>. Reversible myopia is a problem normally seen due to oxygen toxicity<sup>312</sup>. Animal studies showed development of cataract after prolonged HBOT treatments<sup>314-316</sup>.

Several clinical trials were conducted to assess the role of HBOT in the treatment of diabetic foot ulcers. Even though most of the studies reported positive effect some methodological issues preclude a clear conclusion. A lot of studies were retrospective or when they were performed prospectively they were non-blinded or unclear randomized<sup>317</sup>. However a recent monocentric, double blinded and clearly randomized study points out on improved wound healing and improved quality of life after one year<sup>313, 318</sup>. Even though the other prospective studies with enough quality to be taken in consideration suggest a superior effect of HBOT on diabetic wounds they show absence of variation in the control group (no healing)<sup>319,320</sup> that contribute to a high heterogeneity (I=85%) that preclude observation of a positive effect after one year<sup>321</sup>. It is therefore a big need for additional clearly defined studies with more participants that could define the exact subgroup of patients that will benefit most from the HBOT.

## 2 AIMS

The overall aim of the work presented in this thesis was to investigate specific pathogenic mechanisms that contribute to the defective wound healing in diabetes in order to suggest potential new therapeutic targets.

Specific aims

- To identify intracellular pathways modulated by high glucose levels with potential relevance for wound healing processes.
- To study the mechanisms by which glucose contributes to HIF and Notch modulation
- To study the potential therapeutic effect of HIF and Notch modulation for diabetic wound healing
- To study the effect of HBOT on HIF signaling and the therapeutic relevance for diabetic wound healing.



### 3. MATERIALS AND METHODS

**Animals:** C57BL/KsJm/*Leptdb*(db/db) diabetic mice and their heterozygotes control non-diabetic littermates (age 14-20 weeks) were originated from breeding pairs obtained from Charles River (Belgium) (Paper I, II, IV). Db/db mice represent a commonly used model to study type 2 diabetes complications. Due to a deficient leptin signaling, these mice become hyperphagic with subsequent obesity, hyperglycemia and dyslipidemia after 8 – 10 weeks of age<sup>322</sup>.

Skin specific Notch 1 knockout mice (Paper II) were generated from crossing *NI<sup>flox</sup>/NI<sup>flox</sup>* females *KRT14-Cre* males (Breeding pairs obtained from Charles River, Belgium), and in F1, *NI<sup>flox</sup>/+*; *KRT14-Cre*/+ male offsprings were backcrossed to unrelated *NI<sup>flox</sup>/NI<sup>flox</sup>* females. The F2 *NI<sup>flox</sup><sup>flox</sup>*; *KRT14Cre*+/+ mice displayed typical hair phenotypes (without hair) but not all the other offsprings (*NI<sup>flox</sup>/+*; *KRT14-Cre*/+, *NI<sup>flox</sup>/NI<sup>flox</sup>* or *NI<sup>flox</sup>/+*). Heterozygous *NI<sup>flox</sup>/+* were used as controls. Female BALB/C mice, 6 to 8 weeks old were used for Dll4 vaccination experiments (Paper III). All animals were maintained under controlled light and temperature, with free access to standard food and water. The experimental procedure for animals was approved by the North Stockholm Ethical Committee for care and use of laboratory animals.

**Streptozotocin Induced Diabetes:** Diabetes was induced in *NI<sup>flox/flox</sup>*; *KRT14Cre*+/+ mice and Heterozygous *NI<sup>flox</sup>/+* by streptozotocin (STZ) according to the instructions from AMDCC (*Animal Models of Diabetic Complications Consortium*). Briefly, the animals (8-10 weeks) received 50mg/kg STZ mixed in sodium citrate buffer (i.p) daily for five consecutive days. All the treated mice became diabetic after 2 weeks from the first STZ injection. Animals were kept diabetic for three weeks before the start of wound healing experiment (13-14 weeks).

**Wound Model:** Following blood glucose control, general anesthesia was performed with 3% isoflurane (Abbott). The hair of the back was shaved with an electric clipper followed by a depilatory cream. The skin was rinsed with alcohol and two full-

thickness wounds extending through the panniculus carnosus were made on the dorsum on each side of midline, using a 6-mm biopsy punch. A transparent dressing (Tegaderm; 3M) was applied to cover the wounds after topical application of drugs: Paper I: 100  $\mu$ l of DMOG (2 mM), DFX (1 mM), or vehicle alone, Paper I, IV: four injections of 20  $\mu$ l of the viral suspension ( $10^9$  pfu/ml) containing HIF-1 V-N, HIF-1 V-NC, or LacZ-expressing adenoviruses were injected intradermally into the wound edges using a 30-gauge needle, Paper II 100  $\mu$ l of DAPT (100  $\mu$ M), L-685, 458 (100  $\mu$ M) or DMSO control, and in STZ induced diabetic Notch1 knockouts. Following the surgical procedure, the animals were individually housed. During the first 2 days, the animals received s.c. buprenorphine (0.03 mg/kg) twice a day for relief of any possible distress caused by the procedure. In the experiments aimed to analyze histology, mRNA, or protein expression, the wounds were harvested at 7 days after surgery ( $\approx$ 50% closed). Freshly made treatment was applied through the dressing using a 30-gauge needle every other day. Viruses (HIF-VN, HIF-V-NC, LacZ) were inoculated once at the beginning of the experiment, all the other treatments every other day. Each treatment was evaluated in 10 animals per group.

**Wound Analysis:** Digital photographs were recorded at the day of surgery and every other day after wounding. A circular reference was placed alongside to permit correction for the distance between the camera and the animals. The wound area was calculated in pixels with ImageJ 1.32 software (National Institutes of Health), corrected for the area of the reference circle and expressed as percentage of the original area.

**Tissue Preparation and Histological Analysis:** After fixation in formalin, the samples were embedded in paraffin and sectioned (5  $\mu$ m). For histological evaluation, sections were deparaffinized and rehydrated followed by hematoxylin and eosin staining. All slides were then evaluated by light microscopy by two independent observers unaware of the identity of the biopsy, using a semi-quantitative score to evaluate vascularity, granulation, and dermal and epidermal regeneration as previously described<sup>323</sup> and internally validated in our laboratory. We used four-point scales to evaluate vascularity (1, severely altered angiogenesis with one or two vessels per site and endothelial edema, thrombosis, and/or hemorrhage; 2, moderately altered angiogenesis with three to four vessels per site, moderate edema and

hemorrhage, but absence of thrombosis; 3, mildly altered angiogenesis with five to six vessels per site, moderate edema, but absence of thrombosis and hemorrhage; and 4, normal angiogenesis with more than seven vessels per site with only mild edema but absence of thrombosis and hemorrhage) and granulation tissue formation (1, thin granulation layer; 2, moderate granulation layer; 3, thick granulation layer; and 4, very thick granulation layer) and a three-point scale to evaluate dermal and epidermal regeneration (1, little regeneration; 2, moderate regeneration; and 3, complete regeneration).

***Immunohistochemistry staining and Evaluation:*** We evaluated microvessel density by semi-quantitative, double-blind analysis of the specific binding of GS-1 isolectin B4 to microvascular structures using a four-point scale (0, no positive vessels; 1, low number of positive vessels; 2, moderate number of positive vessels; and 3, high number of positive vessels). Isolectin B4 binding was performed using biotinylated isolectin B4 (diluted 1:25). Expression of the adenovirus-mediated transfer of  $\beta$ -galactosidase was evaluated by immunohistochemistry using anti- $\beta$ -galactosidase antibody (1:500) from Abcam. Matched IgG isotype controls were included for each marker. In paper I, the hypoxia level within the wounds was evaluated using the Hypoxiprobe kit (Natural-Amersham Pharmacia) following the instructions of the manufacturer.

***Cell Culture:*** Primary Human Dermal fibroblasts (HDFs) (Promocell, Germany), Mouse embryonic fibroblasts (MEFs) (kindly offered by Dr. Daiana Vasilcanu, Cancer Centrum Karolinska), Primary mouse skin fibroblast cultures (prepared as described in the next section) - HDFs, MEFs and 3T3 cells were cultured in DMEM (5.5 mM glucose) supplemented with 2mM L-glutamine, 100 IU/ml penicillin and streptomycin, and 10% heat-inactivated FBS (Invitrogen). Human Dermal Microvascular endothelial cells (HDMECs) purchased from Promocell, were cultured in the ready to use medium provided by Promocell. All the cells were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C in a cell culture incubator. Only cells between passages 4 to 9 were used. The human SKRC-7 cell line, originating from renal carcinoma from a patient with point mutated VHL, was kindly provided by E. Oosterwijk (Nijmegen, The Netherlands) and maintained as described<sup>324</sup>.

***Establishing fibroblast primary cell culture:*** Primary mouse skin fibroblasts were established by skin explant technique<sup>325</sup>. The cells were maintained in a humidified

atmosphere with 5% CO<sub>2</sub> at 37 °C DMEM (5.5 mM glucose) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin, and 10% heat-inactivated FBS. Only cells between passages 4 and 9 were used (Paper I).

**Hypoxia treatment:** Cells were incubated in a Modular Chamber (Billups-Rothenberg) which is flushed with a gas mixture (95% N<sub>2</sub> and 5% CO<sub>2</sub>) until the oxygen concentrations lowered to 1%, oxygen levels were constantly measured and monitored by an oxygen meter (Dräger) (Paper I).

**Activation of Notch signaling using Recombinant ligands:** Cell culture plates were first coated overnight with Protein G (Invitrogen) 50 mg/ml in PBS at room temperature (RT) and then washed two times with PBS and blocked with 3% BSA in PBS for 2 h at RT. Plates were washed again two times with PBS and incubated with either recombinant Jagged1-FC chimera, 5.8 µg/ml (Ct.No – 599-JG from R&D systems) or Dll4-CF, 2 µg/ml (Ct.No-1389-D4/CF) for 2–4 h at RT. After washing two times with PBS, cells (MEFs and HDFs) were immediately plated and then grown in DMEM containing either 5.5mM or 30mM glucose (Paper II).

**Reporter Gene Assay:** In Paper I, 3T3 cells were transfected with 500 ng of a GAL4-driven luciferase reporter gene plasmid and 10 ng of NTAD residues (pFLAG-GAL4-mHIF1α-[531–584]) or CTAD residues (pFLAG-GAL4-mHIF1α-[772–822]) using Lipofectamine 2000 (Invitrogen) in 60-mm plates, following the instructions of the manufacturer. In Paper II, HDFs placed on Jagged1 coated plates were transfected with 500 ng of 12XCSL luciferase reporter gene plasmid (kindly offered by Dr. Teresa Pereira, CMB) using Lipofectamine 2000 (Invitrogen) in 100-mm plates, following the instructions of the manufacturer. In Paper IV, Transcriptional activity of HIF-1α was assayed using a plasmid (pT81/HRE-luc) containing hypoxia responsive element (HRE) from erythropoietin. HDFs were co-transfected in 12 wells plates with 500ngs of HRE plasmid and 25ngs of Renilla plasmid (used for normalization) (Promega) using Lipofectamine 2000 in Optimem (Life Technologies) for 3h, when the medium was changed to regular cell culture medium (DMEM with 10% FBS). After exposure to the planned experimental conditions, the luciferase activity was assayed (BioThema) in the cell extract and expressed relative to the total protein concentration as evaluated by Bradford method (Bio-Rad) (as described in the Paper I, II accordingly) or relative to Renilla activity (as described in the Paper IV).

**RNA interference experiments:** In Paper I, Human dermal fibroblasts were transiently transfected with 200 pmol per well of either pVHL-siRNA (Hs\_VHL\_5 HP validated siRNA SI02664550 for gene pVHL) or scrambled siRNA from Qiagen using HiPerFect. transfection reagent (Qiagen). After 48 h, cells were exposed to different glucose concentrations (5.5 mM and 30 mM) for another 48 h when the RNA was prepared as described. In Paper II, siRNA oligos against Notch 1,3 and 4 were obtained from Sigma (SASI\_Mm01\_00104901 N1, SASI\_Mm01\_00057178 N3, SASI\_Hs01\_00052678 N4). All stars negative control (scrambled) siRNA from Ambion was used as a scrambled siRNA control. All siRNAs were reconstituted under RNase-free conditions according to the manufacturer's protocol, using the buffers supplied. HDFs or HDMECs were transfected with the 20nM siRNAs at a confluence of 80–90% with HiPerFect Transfection Reagent (Qiagen) in OPTIMEM-1 medium (GIBCO) according to the supplier's protocol. Transfected cells were trypsinised and used for either migration assay or angiogenesis assay

***In vitro* Migration assay:** The cell migration was studied using the “*in vitro* scratch” assay as described<sup>326</sup>. Briefly, HDFs were plated in 12 wells cell culture plates that were pre-coated with collagen (50 µg/ml) and blocked with BSA (3% BSA in PBS). After reaching confluence, the cells were serum starved (overnight) and a scratch was performed with a micropipette tip on the following day in each well. After rinsing with PBS the HDFs were incubated for additional 16 hours with a gamma secretase inhibitor (10 µM DAPT or 10 µM L-685,458) or with control (DMSO) dissolved in DMEM supplemented with 0.2% FBS with different glucose concentrations (5.5mM or 30mM). Mitomycin C (10 µg/ml) was included in the media to prevent cell proliferation. Pictures were taken immediately after scratching (basal level) and after 16 hours with a digital camera coupled to an inverted phase microscope. The relative migration of the cells was calculated from the area measured 16 hours after scratching relative to the basal area expressed in pixels, using ImageJ 1.32 (N.I.H., USA) software. The results were expressed as percentage from the migration of the cells grown in 5.5mM and exposed to control (Paper II).

***In vitro* Angiogenesis Assay:** HDMECs were seeded at a density of  $1 \times 10^4$  cells/well in 150  $\mu$ l culture medium in a 96-wells plate pre-coated with 50  $\mu$ l EC-Matrigel/well (Chemicon; Cat. No. ECM625). The tube formation was quantified 12h after the treatments (DMSO, DAPT, VEGFA or siRNA specific to Notch 1 and 4) by counting the number of sprouting tube-like structures at randomly 5 selected fields under an inverted phase contrast microscope at  $40 \times$  magnifications. Three independent experiments were performed, and the data was presented as the total number of tubes formed in each experiment (Paper II).

***Hyperbaric oxygen treatment:***

For the *in vitro* experiments the cells were exposed to HBO (100% oxygen at 2.5bar absolute pressure) for 1hr in a hyperbaric oxygen chamber while the cells used for control were simultaneously placed outside the chamber. For the *in vivo* experiments the animals were placed in an animal hyperbaric oxygen chamber (RSI-B11; Reimers Systems) and exposed to HBO at 2 bar abs for 90-min. The untreated control mice, were placed outside the hyperbaric chamber in the same room. HBOT session for *in vivo* experiments begins with a progressive increase in pressure for 15min, followed by 60min of continuous exposure to 100% oxygen at 2 bar absolute. After 60min of exposure the pressure in the chamber is slowly reduced during a 15min period. After the procedure the animals were placed in single cages in the animal care room.

***<sup>3</sup>H-thymidine incorporation assay:*** MEFs HIF<sup>+/+</sup> and MEFs HIF<sup>-/-</sup> cells were plated at a density of  $2 \times 10^4$  cells/well in a 6 wells plate. Cells were starved overnight and then exposed for two successive exposures to HBO for 60min (as described in hyperbaric oxygen treatment). After 24 h from the last HBO exposure 1  $\mu$ Ci/ml of <sup>3</sup>H-thymidine (PerkinElmer, Boston, MA, USA) was added to each well. Four hours later the cells were washed twice with cold PBS and then with cold 5% TCA followed by solubilization with 0.5 N NaOH. The solubilized cells (400  $\mu$ l) were mixed with 4 ml scintillation liquid and counted in a beta counter (Packard BioScience, Downers Grove, IL, USA).

***Western Blotting:*** Western Blot analysis was performed for evaluating HIF (Paper I & IV), Notch 1 intracellular domain (Paper II) and expression of plasmid vaccine protein (Paper IV). Proteins from cells or skin (extracted using 2-mm Zirconia beads and a mini-



bead beater (Biospec Products)) were extracted using RIPA Buffer in the presence of protease inhibitors as described<sup>168</sup>. The same amount of protein was loaded in SDS gels (7%), separated by electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked in 5% milk and further incubated first with the primary antibody for 3hrs in 0.1% Tween PBS, and then incubated with HRP conjugated anti-goat secondary antibody (Thermo Scientific, 1:3000). After extensive washing with PBS the complexes were visualized using enhanced chemiluminescence (GE Health Care) according to the manufacturer's instructions.

***Quantitative Real time – PCR:*** Total RNA was isolated from cells using RNeasy RNA extraction kit (Qiagen), and from skin using an RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was obtained by reverse-transcribing total RNA with SuperScript III and first-strand synthesis Supermix for quantitative RT-PCR according to the manufacturer's recommended protocol (Invitrogen). The primers (as described in papers I, II, IV) designed by using the Primer 3 program (<http://frodo.wi.mit.edu>) or chosen from Harvard primer bank (<http://pga.mgh.harvard.edu/primerbank/>). Real-time PCR was performed in an Applied Biosystems 7300 or 7900 units using Platinum SYBR Green quantitative PCR Supermix-UDG with ROX reference dye (Invitrogen). After incubation for 2 min at 50 °C and 2 min at 95 °C, a two-step cycling protocol (15 s at 94 °C, 30 s at 60 °C) was used for 40 cycles. The melting curve analysis was done using the program supplied by Applied Biosystems. The quality of the quantitative PCR run was determined by standard curves and melting curve analysis. The amplification products were verified by sequencing.

***Statistical analysis:*** Differences between groups were computed using one-way analysis or two way repeated measures of variance (ANOVA) as appropriate, with *Bonfferoni* post hoc test. A  $p < 0.05$  was considered statistically significant.

## 4 RESULTS AND DISCUSSION

Diabetic foot ulcer represents a major complication of diabetes that may lead to amputation. For the moment, there is no specific therapy available for diabetic foot ulcers and it has become a priority to develop novel rational therapeutic strategies based on new pathophysiological mechanisms. Our focus was therefore to delineate relevant pathogenic pathways specifically deregulated in diabetes that could contribute to the defective wound healing.

### 4.1 High glucose modulates cellular pathways with potential relevance for wound healing

*(Paper I and Paper II)*

The central pathogenic factor for development of complications in diabetes is high glucose concentration. During the last decade hypoxia has also started to be recognized as an important pathogenic contributor to chronic complications of diabetes. Major findings in the present thesis consist of the description of two new cellular pathways with high relevance for wound healing that are specifically modulated by hyperglycemia and contribute to defective wound healing in diabetes i.e. repression of HIF- $\alpha$  pathway in hypoxia (**Paper I**) and induction of Notch Signaling (**Paper II**).

We first focused on the modulation of HIF-1  $\alpha$  in two cell types, essentially affected during development of chronic complications of diabetes i.e. primary human dermal fibroblasts (HDFs) and human dermal microvascular endothelial cells (HDMECs). In normoxia, we could not detect any influence of high glucose concentrations on HIF-1  $\alpha$  expression. However, the hypoxia-stabilized HIF-1  $\alpha$  is impaired by glucose as previously described by our group and others<sup>327-330</sup>. The repressive effects of high glucose on HIF-1  $\alpha$  stabilization were described in other primary cells and tissues also but are different in transformed tumor cells pointing out on its specificity for diabetes (data not shown).

High glucose destabilizes HIF-1  $\alpha$  as early as after 6hrs which highlights the potential relevance for the immediate cell reaction to hypoxia in acute ischemic events (acute myocardial infarction, stroke). The HIF repression induced by hyperglycemia



continues even at later time points with potential significance for chronic complications of diabetes since most of the tissues prone to develop complications share a hypoxic environment (kidney, nerves, retina etc.).

In order to further understand the intimal mechanism behind the effect of hyperglycemia on HIF-1 alpha stability we investigated the potential involvement of pVHL dependent degradation mechanism, we could appreciate that hyperglycemia contributes to HIF-1alpha degradation through a pVHL-dependent mechanism since HIF-1 is no longer modulated by hyperglycemia in renal carcinoma cells that lack functional pVHL and traditional target genes as VEGFA are no longer modulated in HDF if VHL is specifically silenced. However, VHL expression is not induced by hyperglycemia suggesting that hyperglycemia just increases the sensitivity of HIF-1 $\alpha$  to VHL dependent degradation. The same VHL dependent degradation modulated by glucose was also observed in myotubules but not in tumoral cells<sup>329</sup>.

In concordance with the VHL mediated degradation of HIF-1alpha in hyperglycemia PHD inhibitors are able to reverse the repressive effect of glucose. The negative regulatory effect of glucose was not only restricted to the stability of HIF-1alpha but also affected both HIF-1 alpha transactivation domains NTAD and the CTAD. In agreement with our results, it has been shown that hyperglycemia induced decrease in transactivation of HIF-1 alpha and limited the HIF-1 alpha function<sup>331</sup>. The repressive effect of hyperglycemia on both HIF-1alpha stability and transactivation was mirrored by down-regulation of several HIF-1 $\alpha$  target genes essential for wound healing such as heat shock protein 90 (HSP-90), VEGF-A, VEGF-R1, stromal cell-derived factor (SDF)-1 $\alpha$ , and stromal cell factor (SCF) .

The Notch signaling pathway is involved in many cellular processes with potential relevance for wound healing as cell differentiation, cell migration, proliferation, angiogenesis etc<sup>245,249,332,333</sup>. In **Paper II**, we have observed activation of Notch signaling in skin of different diabetic animals, as assessed by relative mRNA expression of several Notch target genes. We have therefore investigated if high glucose has direct effect on Notch signaling in HDFs and HDMCEs. Exposure to high glucose concentrations induces indeed the active intracellular domain (NICD) which is followed by Notch mediated transcriptional activation as shown by induction of the highly Notch

specific 12XCSL-luc reporter assay. Hyperglycemia induces the Notch signaling at a level before the cleavage of Notch receptor since treatment of the mouse embryonic fibroblasts (MEFs) with gamma-secretase inhibitor (DAPT) cancel the stimulatory effect of high glucose concentration on Hey1. Moreover the stimulatory effect of hyperglycemia on Notch signaling is not restricted to the fibroblasts but affects also other cells with important roles in wound healing e.g. human dermal microvascular endothelial cells (HDMEC) where several Notch-related genes (*Notch1*, *Notch4*, *Hes1*) were induced by exposure to high glucose concentrations. The level of glucose inducing effect on Notch signaling is common in different tissues since  $\gamma$ -secretase inhibitors are able to cancel the effect of hyperglycemia on functional assay performed both in HDF (migration assay) and in HDMEC (in vitro angiogenesis). Modulatory effects of hyperglycemia on Notch signaling were found as well in podocytes <sup>276</sup> and in neural stem cells<sup>334</sup>.

#### 4.2 Glucose affects HIF-1 alpha function by different mechanisms

High glucose modulates HIF-1 stability and function at multiple levels. The effect is however restricted to posttranslational level (figure 6) since HIF-1 alpha RNA were not influenced by High glucose concentrations in either hypoxia or normoxia.

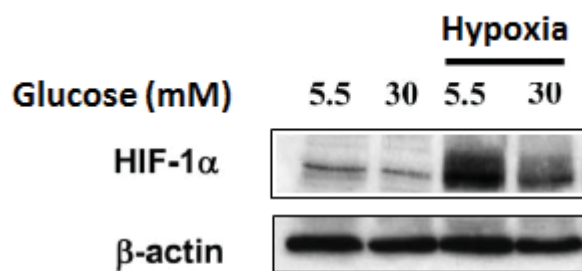


Figure 6: Hyperglycemia Impairs HIF-1 alpha stability in mouse dermal fibroblasts

Hyperglycemia modulates HIF-1 alpha degradation since it has been shown that the effect of high glucose on HIF-1alpha stability is cancelled if the cells were exposed to the proteosomal inhibitor, MG132 <sup>327</sup>. Moreover we could establish that the effect of glucose is mediated by a pVHL dependent mechanism. It is however an increase of the interaction between VHL and HIF-1alpha which is activated by hyperglycemia since

VHL levels are not modulated by glucose. In concordance with the central importance of prolyl hydroxylation for facilitating the interaction between HIF-1 $\alpha$  and VHL prolylhydroxylases (PHD) inhibitors (DMOG and DFX) are able to rescue at least partially the effect of hyperglycemia on HIF-1  $\alpha$  stability and function both in normoxia and in hypoxia. However, it should be stressed that HIF-1  $\alpha$  destabilization in high glucose is only partially reversed by the PHD inhibitors suggesting the role of other mechanisms, in addition to proline hydroxylation, as already suggested before<sup>327</sup>.

Methylglyoxal is a reactive  $\alpha$ -oxoaldehyde produced in the cells exposed for long time to hyperglycemia that was reported to modulate HIF-1 reaction to hypoxia. Methylglyoxal modifies HIF-1 $\alpha$  at two specific residues, arginine 17 and arginine 23, and reduces its interaction with HIF-1 beta.<sup>335</sup> Moreover methylglyoxal affects p300 interaction with HIF-1 $\alpha$  and interferes with HIF-1 transactivation. An important role was identified in one residue in p300 since mutation of arginine 354 of p300 restored its interaction with HIF-1 $\alpha$  in presence of high-glucose<sup>331</sup>. Methylglyoxal increases HIF-1 $\alpha$  degradation by increasing its interaction with molecular chaperones CHIP, HSP40 and HSP70 which eventually leads to polyubiquitination of HIF-1 $\alpha$  and its degradation<sup>336</sup>. Methylglyoxal leads to accumulation of CHIP, Hsp40 and decreases the levels of the molecular chaperone Hsp90. It is however a more complicated interaction in the presence of hyperglycemia since CHIP and HSP 70 contribute to HIF-1 $\alpha$  degradation but not HIF-2 $\alpha$  degradation<sup>337</sup> which is in discordance with our observation that hyperglycemia destabilizes HIF-2 $\alpha$  to the same extent as HIF-1 $\alpha$ . Interestingly, Methylglyoxal decreases Hsp90<sup>338</sup> and can modulate HIF-1 $\alpha$  stability by this way since Hsp90 has an established role for stabilization of HIF-1  $\alpha$ <sup>108,339</sup>.

Several other factors could play a significant role in the destabilization of HIF in presence of high glucose. For example, p53 is able to interfere with HIF-1  $\alpha$  stability and activity by a pVHL independent mechanism<sup>340</sup> and it is induced by high glucose<sup>341</sup>. However HIF-1  $\alpha$  is still destabilized by high glucose in fibroblasts deficient of p53<sup>327</sup>. Sumoylation is an important process in HIF-1  $\alpha$  stability, RSUME an inducer of SUMOylation increase SUMO conjugation with HIF-1  $\alpha$  and stabilize HIF-1  $\alpha$ <sup>115</sup> during hypoxia. Sirtuins regulates protein functions by sumoylation and

SIRT1 sumoylation regulates activity of HIF-1 alpha and participates in HIF-1 alpha accumulation and transcriptional activity under hypoxia<sup>342</sup>. High glucose reduces SIRT1 expression levels and enzyme activity<sup>343</sup> making SIRT1 a potential mediator of hyperglycemia on HIF-1 alpha destabilization in diabetes.

By investigating mediators of hyperglycemia on HIF function we will be able to identify new specific therapeutic targets to rescue the repressed reaction of the cells to hypoxia characteristic in diabetes.

#### ***4.3 HIF-1 alpha stabilization improves the defective wound healing in diabetes (Paper I & IV)***

To assess the *in vivo* significance of the modulation of HIF by hyperglycemia we have further studied the modulation of HIF-1 in experimental diabetic wounds.

In perfect agreement with the *in vitro* results, a general repression of HIF-1 signaling was recorded in wounds in diabetic mice (db/db) despite profound hypoxia as evaluated by pimonidazol staining. We observed both lower levels of HIF-1 alpha expression and lower levels of HIF target genes with relevance for wound healing (**Paper I**). These data are in agreement with previous reports in patients with diabetic chronic foot ulcers that express lower levels of HIF-1alpha compared with patients with chronic venous ulcers despite the same levels of hypoxia<sup>327</sup>. The same destabilization of HIF-1alpha in diabetic skin was also reported by others<sup>344</sup>. Moreover, the fibroblasts isolated from the skin of diabetic patients are unable to induce VEGFA in response to hypoxia exactly like fibroblasts from diabetic mice<sup>331</sup>. We also observed the same inappropriate HIF reaction to hypoxia in diabetic kidneys<sup>345</sup>.

Low oxygen levels as a consequence of macro- and micro- angiopathy are present locally in patients with diabetic foot ulcers or diabetic neuropathy<sup>346-349</sup>. Based on our *in vitro* and *in vivo* observations on the repression of HIF-1 signaling by hyperglycemia we hypothesized that in diabetes the defect of the cells to adapt to hypoxia might play a central role in development of chronic complications. To test this hypothesis we choose to study the therapeutic potential of HIF-1 alpha induction for wound healing in db/db mice (**Paper I**) that are the best rodent model for wound healing defects in diabetes<sup>350</sup>.

For inducing HIF-1 in the wounds we have used either compounds that interfere with activity of HIF hydroxylases, by chelating  $\text{Fe}^{2+}$  (DFX) or by competing with 2-oxoglutarate (DMOG) (**Paper I**) or by hyperbaric oxygen therapy (HBOT) (**Paper IV**). All the above treatments were able to induce HIF-1 alpha accumulation, and expression of HIF target genes essential for wound healing (HSP-90, VEGF-A, VEGFR1, SDF-1, and SCF).

Local application of HIF hydroxylase inhibitors (DMOG and DFX) or treatment with HBO improved the healing process in db/db mice despite the presence of persistent chronic hyperglycemia (Paper I and IV). However to obtain direct evidence of HIF-1 role in the diabetic wound healing, we performed a gain of function studies with adenoviruses expressing stable forms of HIF-1 alpha (V-N and V-NC) in which both the critical proline residues have been substituted with alanine's. Indeed local injection of adenoviruses containing stable HIF-1 alpha around the wound edges improves the wound healing rate in db/db mice confirming the central role of HIF-1 repression as pathogenic defect in diabetic wounds. The positive effect of local HIF induction for wound healing in diabetes was shown in other experimental designs as well using either chemicals or virus mediated transfer<sup>331,344</sup>. The functional consequence of HIF hydroxylases on diabetic wounds improved several processes important for healing (i.e., granulation, vascularization, epidermal regeneration, and recruitment of endothelial precursors).

#### ***4.4 Diabetes Impairs of HIF-1 alpha regulation in several tissues***

There is a large body of evidence supporting that HIF-1 $\alpha$  is destabilized by high glucose concentrations. Moreover inappropriate low levels of HIF-1 are found in diabetes in several tissues with negative consequences. Biopsies from patients with diabetic foot ulcers showed decreased HIF-1 alpha levels compared to patients with venous ulcers that share the same hypoxic environment but are not exposed to hyperglycemia<sup>327</sup>. The functional relevance of this observation was confirmed by other groups beside us that have also shown that gene-based therapy with HIF-1alpha induced acceleration of wound healing and angiogenesis in diabetic mice<sup>330,344</sup>.

Diabetes impairs through HIF-1 hypoxia-induced production of SDF-1, CXCR4, VEGF and eNOS and lead to endothelial dysfunction. Hyperglycemia represses HIF-1

alpha and is followed by an increase in the myocardial infarct size in rats<sup>351</sup>. The same dysfunction of HIF-1 in presence of hyperglycemia is followed by decreased angiogenesis in diabetic patients<sup>352,353</sup>. However overexpression of HIF-1 normalized VEGF levels and improved myocardial capillary network following myocardial injury in diabetic mice<sup>354</sup>.

In addition, increased expression or stabilization of HIF-1 $\alpha$  is critical to increase limb perfusion and function in diabetic mice, along with an increase in the number of circulating EPCs and vessel density<sup>355</sup>. A polymorphism of HIF-1alpha (P582S) that confers relative resistant to the repressive effect of hyperglycemia is associated with protection against nephropathy in patients with type2 diabetes<sup>345,356,357</sup>

HIF is suggested to play an important role for the function of beta cells in diabetes as well. Several opposing data concerning the effect of the manipulation of the HIF-1 system in beta cells are available<sup>358,359,360,361,362</sup> suggesting that both excessive and inappropriate HIF-1alpha is deleterious for insulin secretion<sup>362</sup>. A polymorphism in HIF-1alpha was associated with type 2 diabetes<sup>345,356,357</sup>.

#### ***4.5 Blocking overactive Notch signaling improves wound healing in diabetes (Paper II & III)***

Overactive Notch signaling *in vivo* confirmed our *in vitro* data. Notch signaling is overactive in the skin of different model of diabetes both in mice and in rat as reflected by induction of several Notch target genes (**Paper II**). Using a specific antibody against the active Notch 1 intracellular domain we could confirm an intense expression of NICD in the granulation tissue of the diabetic wounds (Figure 7).

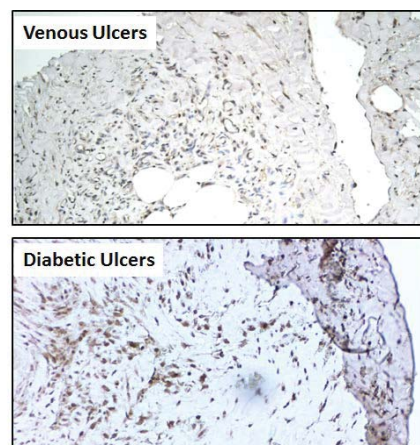


Figure 7. Notch-1 intracellular domain expression in venous and diabetic



To unravel the pathophysiological role of overactive Notch for the diabetic wound healing, we have blocked Notch signaling using gamma secretase inhibitors (DAPT and L-864, 548) in experimental wounds performed in db/db mice. Both compounds improved significantly the wound healing process highlighting the pathogenic role of glucose-stimulated Notch signaling (**Paper II**). Moreover, the positive effects of blockage of the Notch signaling on wound healing seem to be specific for diabetes. We could not observe any effect of the gamma secretase inhibitors (**Paper II**) or Dll4 DNA vaccine (**Paper IV**) on wound healing rate in non-diabetic mice that confirms previous observations<sup>363,364</sup>. Discordant effects were observed in other wound healing models as corneal epithelial wound healing<sup>365 366</sup> that might be a consequence of effectivity of the manipulation of the Notch signaling<sup>367</sup>.

Blocking the overactive Notch signaling in wounds increased the angiogenesis as assessed by isolectin staining and markers of tip cell formation and angiogenic sprouts as assessed by relative expression of vascular endothelial growth factor receptor 2 (VEGFR-2), Platelet derived growth factor B (PDGF-B)<sup>368</sup> and vascular endothelial growth factor receptor 3 (VEGFR-3)<sup>369</sup> (**Paper II**). Moreover, treatment with DAPT is followed by an increase in the expression of chemokines with essential roles in the recruitment of endothelial precursor cells as SDF-1<sup>370</sup> and SCF<sup>371</sup>. Taken together gamma secretase inhibition in diabetic wounds improves the diabetes-dependent repression of granulation and angiogenesis at multiple levels.

These effects were indeed confirmed *in vitro* by blockage of Notch signaling in HDF or in keratinocytes (**figure 8**) by exposure to gamma secretase inhibitors (DAPT and L-685,458) rescued the inhibitory effect of hyperglycemia on cellular migration. Moreover, the same treatment increased the *in vitro* angiogenesis in HDMECs. However, the positive effects of Notch inhibition on migration and angiogenesis are mediated exclusively through Notch1 receptor, since Notch1 specific siRNA but not Notch3, Notch2 or Notch4 siRNA mimics the effects of gamma-secretase inhibitors (**Paper II**).

To investigate the role of Notch1 receptor in diabetes repressed wound healing *in vivo*, we studied the wound healing rate in skin specific Notch1 knockout mice in which diabetes was induced by streptozotocin. Diabetic skin specific Notch1 knockout mice

have a significantly improved healing rate compared to its diabetic heterozygote control (**Paper II**) pointing out the importance of Notch 1 receptor in diabetes and its essential role in diabetic wound healing (figure 9).

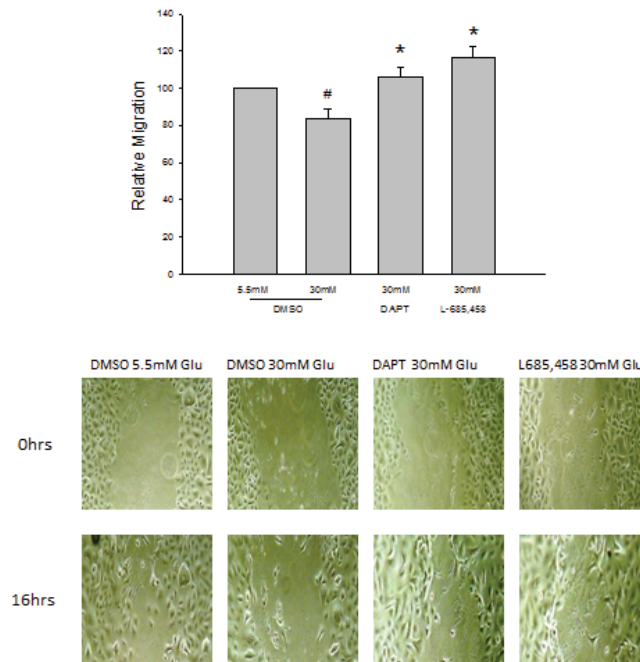


Figure 8. Relative rate of migration in Keratinocytes treated with gamma-secretase inhibitors in presence of Normal (5.5mM) and high glucose (30mM)

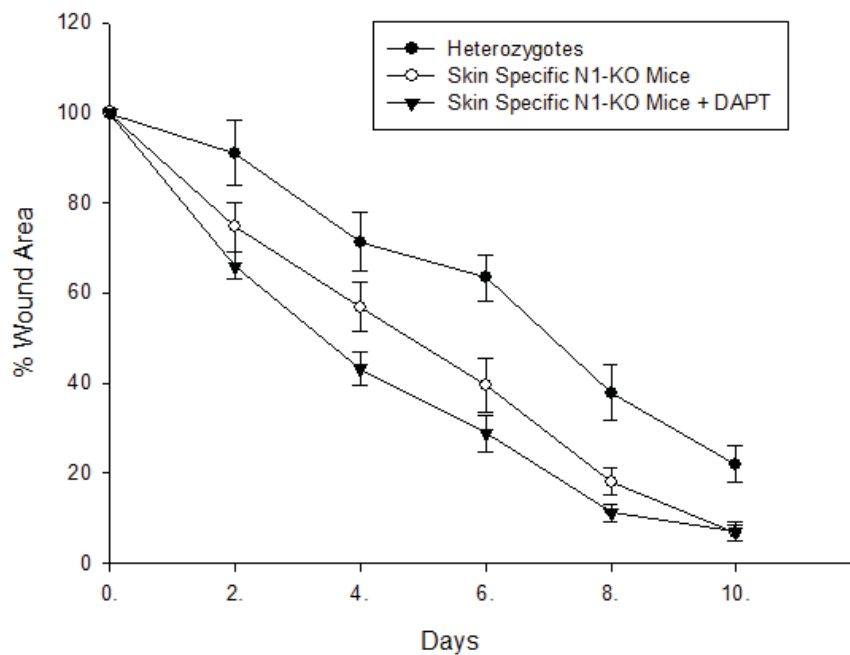


Figure 9. Notch-1 deficiency in skin improves wound healing in diabetes mice, with no additional effect of DAPT on Notch1 knockout mice



#### 4.6 Hyperglycemia induced Notch signaling mechanisms

We here report for the first time that Notch signaling is activated in diabetic skin and this effect is dependent on hyperglycemia. The mechanism behind the activation of Notch signaling in diabetes is still unclear. We have however investigated some potential mechanisms such as fringe and FoxO genes. Fringes (Manic, Lunatic and Radical fringes) are regulators of Notch signaling, by mediating O-fucosylation and O-glycosylation<sup>372,373</sup> of EGF repeats on Notch receptors. These posttranslational modifications modify the specificity of the interaction between receptors to ligands, and modulate Notch activity in a number of tissue specific contexts<sup>191,192,374</sup>. For example, lunatic fringe (Lfng) inhibits the Notch1 and Jagged1 binding and potentiates Delta1 signaling through Notch1, but potentiates both Delta1 and Jagged1 mediated signaling through Notch2<sup>375</sup>. Loss of *Lfng* enhances angiogenic sprouting by decreasing the affinity of Notch1 and Dll4 binding; in the same time, jagged1 competes with Dll4 in binding to Notch and thereby locally enhances angiogenic growth while Mfng glycosylation of Notch further increases the Dll4-Notch signaling<sup>376</sup>. Interestingly we have observed increase in expression of the fringes and Dll4 genes in the skin of diabetic animals (Paper II) that was reproduced by hyperglycemia in vitro (data not shown). Moreover blocking fringes by siRNA treatment in HDMECs reversed the effect of hyperglycemia induced Notch signaling however without been able to identify a specific effect on any of the isolated fringes (figure 10).

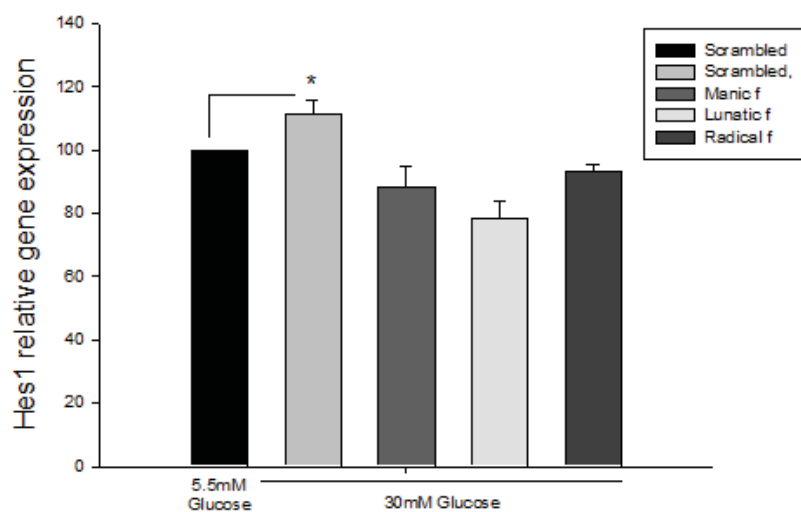


Figure 10. Relative gene expression of Notch target gene, *Hes1* in presence of high glucose in fringe siRNA treated Endothelial cells

FOX (Forkhead box) proteins plays important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity<sup>377</sup>. FoxO1 and Notch interact physically and functionally leading to activation of Notch target genes to regulate the progenitor cell maintenance and differentiation<sup>378</sup>. Recent evidence suggest a synergistic role of FoxO1 and Notch1 on insulin sensitivity. Treatment with gamma-secretase inhibitors improves insulin sensitivity in a FoxO1 dependent manner<sup>274</sup>. This stimulated us to study the potential role of FoxO1 on Notch signaling in our experimental system. We were able to observe that induction of the Notch target gene, Hes1 by hyperglycemia (figure 11) in HDFs is cancelled when FoxO1 was specifically silenced by siRNA suggesting a FoxO1 mediation. However, further investigation is necessary to understand the exact underlying mechanisms of Notch activation by fringes and FoxO.

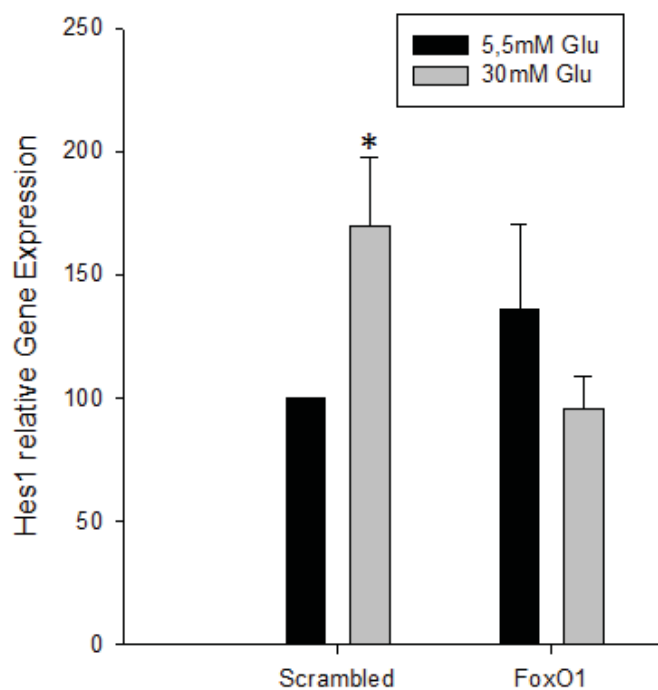


Figure 11. Relative gene expression of Notch target gene, Hes1 in presence of high glucose in FoxO1 siRNA treated Endothelial cells

Notch signaling interacts as well with p53 pathway<sup>379</sup>. NICD1 is activated by p53<sup>276</sup> and p53 is activated by hyperglycemia<sup>341</sup>. It is therefore tempting to propose this interaction as potential mediator of the hyperglycemia effect on Notch signaling.

#### 4.7 HIF-1 alpha and hyperbaric oxygen therapy (HBOT) in treatment of diabetic wounds (*Paper IV*)

Hyperbaric oxygen has been used as an adjuvant therapy for many years in the treatment of complicated diabetic wounds. However, the underlying mechanism is not clear. We show here that HBOT induces HIF-1 alpha stabilization. This effect takes place at later time points after exposure to high oxygen pressure, suggests that is not a direct effect of oxygen but of other different mechanisms. Radical oxygen species (ROS) excess during exposure to HBO could be such a mediator<sup>380</sup> since ROS improve HIF-1 alpha stability and function<sup>381</sup>. It is however difficult to accommodate the ROS impairment of PHDs with the VHL independent regulation noted in HBO exposure. However other potential VHL independent mediators could stabilize HIF in HBOT as HSP90<sup>107</sup>, RACK1<sup>108</sup>, Mdm2<sup>382</sup> Jun activation domain-binding protein-1 (Jab1)<sup>31</sup> FOXO4<sup>383</sup> or GSK3<sup>384</sup>. HBOT effect is via HIF-1 since it stimulated proliferation of wild type MEFs (HIF+/+), but the proliferative effects were abolished in HIF deficient cells (MEFs HIF-/-).

It has been shown that HBO stimulates the release of endothelial progenitor cells (EPC) from bone marrow that finally home in the wounds and contribute to angiogenesis and wound healing<sup>304</sup>. However, this process is defective in diabetes due to low levels of SDF-1 alpha, a cytokine that modulates the EPCs homing. Local application of SDF-1 alpha together with HBOT increases the healing rate in diabetic mice<sup>296,304,370</sup>. Taking into account that SDF-1 is a target gene for HIF-1<sup>370</sup> and the importance of HIF-1 alpha in diabetic wound healing in mice (**Paper I**) we have studied the effect of combined local induction of HIF-1 alpha with HBO treatment. Indeed adenovirus mediated transfer of HIF-1 alpha improves the effect of HBOT on wound healing in db/db mice. In perfect agreement with the central role of HIF-1 alpha as inducer of much more other relevant target genes for wound healing above SDF-1, the transfer of HIF-1alpha was superior to the adenoassociated virus mediated transfer of SDF-1 alpha in potentiating the effects of HBO on wound healing in db/db mice (**Figure 12**).

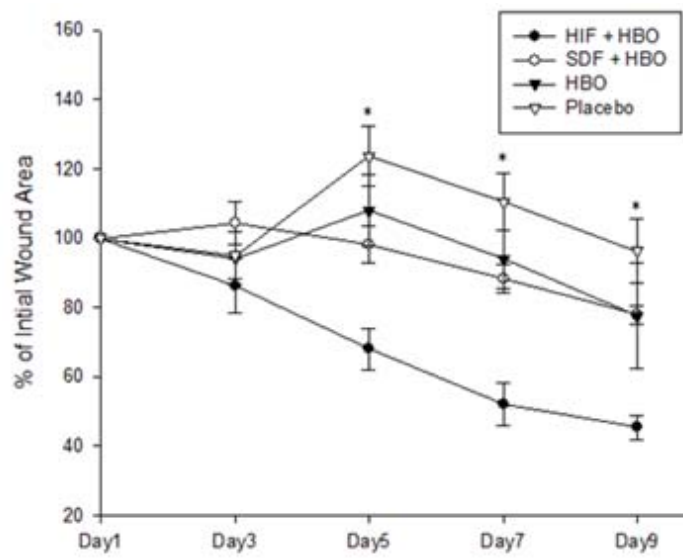


Figure 12. HBO and Adenovirus HIF-1 alpha effects on wound healing in db/db mice

## 5 POINTS OF PERSPECTIVES

The focus of our investigation was to identify new pathogenic mechanisms relevant for the defective wound healing in diabetes. We have identified two new pathways that contribute to the repressive effect of diabetes on wound healing and could propose new therapeutic approaches.

The role of HIF, in diabetes was just emerging when this work started. We have further investigated the mechanisms behind the repressive effect of hyperglycemia on hypoxia induced HIF-1  $\alpha$ . We could show that hyperglycemia destabilizes HIF-1  $\alpha$  through a VHL dependent mechanism- Moreover we could show that hyperglycemia affects both transactivation domains of HIF but stabilization of HIF-1 $\alpha$  is necessary and sufficient for promoting wound healing in a diabetic environment.. We could make the proof of concept concerning the central role of HIF for defective wound healing in diabetes through improvement of healing in in an experimental model of diabetic wounds by using either chemicals that induce HIF or through direct adeno-virus mediated transfer of HIF. These results might offer the premises for conducting clinical studies on wound healing in patients with diabetes. DFX which we used efficiently in vitro and in vivo to induce HIF-1  $\alpha$  is already clinically approved for other indications. Moreover inhibitors of HIF-1 hydroxylases are developed, for other indications by several pharmaceutical companies.

Important future issues are the identification of the critical residues that are sensitive to hyperglycemia-induced HIF-1  $\alpha$  destabilization and to identify the pathways activated by hyperglycemia that lead to HIF repression in diabetes. Both these strategies could suggest more specific future therapies.

Hyperbaric oxygen therapy (HBOT) has been proposed as a medical treatment for diabetic foot ulcers. We demonstrated that HBOT activated HIF-1  $\alpha$  and contribute to diabetic wound healing. Moreover, we could show that local transfer of a stable form of HIF has an additive effect to HBOT improving wound healing in db/db mice. Further studies on the HBOT and HIF-1  $\alpha$  contribution in wound healing in patients with

diabetic foot ulcers will help to further tailor therapeutically use of potential combination between HBOT and HIF alpha local induction.

Notch signaling was identified as another important pathway deregulated by hyperglycemia with relevance for defective wound healing in diabetes. Notch pathway is highly important for several cell fate decisions-. We demonstrated that hyperglycemia could induce Notch signaling both *in vitro* and *in vivo*. Blockage of Notch signaling induced by hyperglycemia using gamma-secretase inhibitors (DAPT or L-658,458) resulted in positive effects on migration and angiogenesis *in vitro*, and improved wound healing in diabetic mice. Moreover, we could show that these effects were specific for diabetes, since treatment with either gamma-secretase inhibitors or DNA vaccine against Dll4 did not influence wound healing in non-diabetic mice. We could also identify the central pathogenic role of Notch1 in Notch dependent defect in wound healing in diabetes through loss of function genetic approaches (siRNA and cre/lox system).

These results offer the premises for the use of gamma secretase inhibitors in clinical studies in patients with DFU, as gamma secretase inhibitors have already been developed and studied in humans, for other indications. However, the potential use of specific Notch 1 inhibitors is of more interest in this context. Having in mind the interplay between HIF signaling and Notch signaling during development it would be highly interesting to further investigate the interaction between these systems in diabetes in general and in diabetes wounds in particular.

## 6 CONCLUDING REMARKS

HIF-1  $\alpha$  represent a potential therapeutic target for improving the defective wound healing process in diabetes:

- High glucose impairs HIF-1  $\alpha$  stability and function *in vitro* and in wounds in diabetic mice
- The repressive effects of high glucose involves a VHL-mediated degradation mechanism
- The increase of local HIF levels through blockade of the HIF hydroxylation or through direct HIF-1  $\alpha$  adenoviral transfer results in improvement of wound healing in diabetes.
- HBOT induces HIF-1  $\alpha$  and the combination of HBOT with local HIF-1  $\alpha$  stabilization reverses the negative effects of diabetic wound healing in mice.
- HIF stabilization is critical for improving defective wound healing in diabetic mice, activating all the essential steps of this process.

Hyperglycemia activates Notch signaling with repressive effect on wound healing in diabetes. Hyperglycemia activates Notch signaling at different levels with negative effects on cell migration and angiogenesis

- Blocking overactive Notch signaling using gamma-secretase inhibitors rescues *in vitro* hyperglycemia repressed migration and angiogenesis, and improved wound healing in diabetic mice
- Notch1 is the main player of the Notch negative effect for wound healing in diabetes

In conclusion, we identified two new pathogenic mechanisms important for defective wound healing in diabetes. Our findings warrant development of specific therapeutics that address HIF and Notch signaling for improving the healing of diabetes wounds.

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