Molecular Mechanisms Underlying the Effect of Hypoxia on Stem Cell Growth and Differentiation
Molecular Mechanisms Underlying the Effect of Hypoxia on Stem Cell Growth and Differentiation

PhD Thesis by

Mayuri Sinha Prasad

Laboratory for Stem Cell Research, Biomedicin, Department of Health Science and Technology, Aalborg University, Aalborg, Denmark

River Publishers
Aalborg
Table of Contents

Preface ................................................. 3
Abstract ............................................. 5
Resumé ................................................. 7
Selected Abbreviations .......................... 10

1  Introduction ........................................... 11
   1.1  Stem cells ........................................... 11
   1.2  Derivation and characterization ............... 12
       1.2.1 Derivation, culture and characteristics of human ESCs .................................................. 13
       1.2.2 Derivation, culture and characteristics of adipose-derived stem cells .................................. 15
   1.3  Role of Oxygen in differentiation, proliferation and ion channel function .................. 18
       1.3.1 The starting point: Hypoxia inducible factor ................................................................. 19
       1.3.2 Regulation of differentiation by hypoxia and Notch cross talk ....................................... 22
       1.3.3 Hypoxia and proliferation .................................................................................................. 25
       1.3.4 Ion channels and their regulation by hypoxia ................................................................. 26
   1.4  Basics of electrophysiology ..................... 27
       1.4.1 Structure and function of ion channels ............................................................................. 27
   1.5  Principle and technique of whole-cell patch-clamp ..................................................... 30
       1.5.1 Historical Background ................................................. 30
       1.5.2 Principle of Patch-clamp Recording .................................................................................. 31
       1.5.3 Patch-clamp application ................................................. 32
       1.5.4 Patch-clamp Configurations ......................................................................................... 33
   1.6  Aim of the thesis ..................................... 36
2 Methods and material.......................................................................................................................... 38

2.1 Semi-quantitative real time PCR and primer designing ................................................................. 38

2.2 Cell culture .................................................................................................................................. 40
  2.2.1 Culturing human ESCs .............................................................................................................. 40
  2.2.2 Culturing human adipose-derived stem cells ......................................................................... 41

2.3 Electrophysiological measurements ............................................................................................ 42

3 Amplification of Nanog and ion channel genes in human embryonic stem cell line
  cultured at 20% and 5% oxygen tensions, with and without Notch suppression ......................... 48

3.1 Introduction .................................................................................................................................. 48

3.2 Method and Material .................................................................................................................... 49

3.3 Results ....................................................................................................................................... 51

3.4 Discussion .................................................................................................................................. 53

4 General discussion and conclusion.................................................................................................. 55

4.1 Maintenance of undifferentiated state by short term and long term exposure to hypoxia 56

4.2 The effect of hypoxia on maintainance of pluripotentiality is reversible ................................. 60

4.3 Molecular mechanisms underlying the maintenance of pluripotency by hypoxia ............... 61

4.4 Culturing human ASCs in mild hypoxia results in alteration of electrophysiological
  properties of the cells in population level. ...................................................................................... 63

5 Strength and limitations of the experiments and future directions ........................................... 66

6 References..................................................................................................................................... 69

7 Appendix ....................................................................................................................................... 84
Paper I: Continuous hypoxic culturing maintains activation of Notch and allows long-term propagation of human ESCs without spontaneous differentiation

Paper II: The effect of human ESCs (human ESCs) long-term normoxic and hypoxic cultures on the maintenance of pluripotency

Paper III: Impact of mild hypoxia on membrane currents and ion channel expression of adipose-derived stem cells
Preface

This thesis is a primary result of four years of work in the Laboratory for Stem Cell Research, Biomedicin, Aalborg University. I would like to thank my former and present colleagues, who have in one way or the other supported me in the realization of this project. I would especially like to thank my supervisor, Trine Fink and also Vladimir Zachar and Cristian P Pennisi for their contribution to the work that form a part of this thesis.

During the span of this study, I have had the opportunity to work with two very different cell types namely, human ESCs and human adipose-derived stem cells. The effect of hypoxia on the cellular functions was the point that brought the study on two cell types together. I have learned several laboratory techniques that helped me reach the goals. Among all the techniques, whole-cell patch-clamping and analysis was the most challenging. With the technique, I have not only learned to handle cells with micro-precision, but have at the same time learned to be extremely patient and perseverant.

This dissertation is presented in the form of collection of scientific papers. It opens with an introduction, followed by description of some important methodologies used. The subsequent 4 chapters are focused on the results obtained during the study and comprise of Paper I, II and III. The thesis closes with a chapter on general discussion and strength, limitations of the experiments and future work within the field.
As I wrap up my PhD work, I would like to thank my entire family for their support during the span of this thesis; and would like to mention that the completion of this work would not be possible without the unabating encouragement from my parent-in laws, Jyoti and Ramjee Prasad, my husband, Rajeev R Prasad and our dearest son Arya. At the end I would like to express that the memories of my parents have been constant source of inspiration and strength. I would like to dedicate this thesis to my parents, Gita and Phanindra Kumar Sinha.

Mayuri Sinha Prasad
Abstract

The potential use of stem cells in regenerative therapy is an exciting field of research, currently undergoing tremendous development in order to bring the applications from the laboratory benches to hospital bedsides. Both embryonic as well as adult stem cells have been shown to be beneficial in the treatment of various debilitating disease in clinical trials around the world.

The main aim of this PhD dissertation is to gain a better understanding of the effect of low oxygen tension on the differentiation, proliferation and ion channel function of human embryonic stem cells (ESCs) and human adipose-derived stem cells (ASCs). For this purpose, the investigation was divided into 3 sub-studies.

The first study focused on identifying the oxygen tension at which the ESCs would attain optimal growth with minimal differentiation along with understanding the underlying molecular pathway responsible for the effect. The results from the investigation indicate that culturing ESCs at 5% oxygen tension can support long term undifferentiated propagation, without locking the cells in a permanent state of pluripotency. Additionally we found that Notch-hypoxia cross talk plays an important role in the maintenance of self-renewal.

The understanding gained from the first study was then taken further by investigating the properties of human ESCs that were subjected to long term hypoxia to evaluate whether low oxygen tension brought about any change in the ESC characteristics like proliferation, expression of differentiation markers, karyotype, telomerase activity, differentiation capacity etc. This study
established that the ESCs exposed to long term hypoxia maintained features associated with stable undifferentiated propagation and at the same time retained the capacity to differentiate into the 3 germ layers.

The aim of the last study was to investigate the difference in the ion channel expression of the embryonic and adipose-derived stem cell cultured in normoxic and 5% hypoxic conditions. The results from this study detected a small but significant change in the level of some ion channel gene expression in ESCs and further showed that hypoxic preconditioning influenced the electrophysiological profile of ASCs on a population level.

Innumerable steps are involved in the transformation of the undifferentiated stem cells into differentiated, functional cells. The understanding of molecular regulators that form a part of this process is absolutely crucial for the development of the regenerative therapy. This dissertation is a small part of the effort to unveil the molecular mechanisms underlying stem cells differentiation, proliferation and ion channel function.
**Resumé**

Den potentielle brug af stamceller i regenerativ terapi er et spændende forskningsområde som for tiden undergår en kolossal udvikling for at bringe resultaterne fra laboratoriet til klinisk brug. Både embryonale og voksne stamceller har vist sig at være gavnlige i behandling af forskellige svækkende sygdomme i kliniske forsøg over hele verden.

Hovedformålet med denne PhD afhandling er at opnå bedre forståelse for effekten af lav iltryk på differentiering, proliferation og ion kanal funktion af embryonale og fedt stamceller. Med dette formål blev undersøgelsen inddelt i 3 understudier.

Det første studie fokuserede på at identificere den iltryk hvor de embryonale stamceller ville opnå den optimale vækst med minimal differentiering samtidig med forståelsen af den underliggende molekylære mekanisme ansvarlig for effekten. Resultaterne af undersøgelsen viser, at dyrkningen af uudviklede stamceller ved 5% iltryk kan understøtte langtids udifferentieret formering uden at fastlåse cellerne på et permanent pluripotent stadium. Samtidig fandt vi at Notch-Hypoxia kryds signaler spiller en vigtig rolle i oprettelsen af selv-fornyelse.

Forståelsen opnået fra det første studium blev så ført videre ved undersøgelse af de egenskaber ved embryonale stamceller som blev underlagt lang tids hypoksi for at evaluere om lav iltryk frembragte nogen forandring i embryonale stamceller karakteristikkerne så som proliferation, differentieringskarakteristika, karyotype, telomerase aktivitet, differentieringskapacitet o.s.v. Studiet slog fast at de embryonale stamceller der blev udsat for lang tids hypoksi beholdt
karakteristika svarende til stabil udifferentieret formering og samtidig beholdt de evnen til differentiering til de 3 kimlag.

Formålet med det sidste studie var at undersøge forskellen på dyrkningen af embryonale og fedt stamceller under normoksiske og 5% hypoksiske betingelser. Resultaterne fra dette studie afslørede en lille men markant forandring i niveauet af ion kanal gen forekomst i de embryonale stamceller og viste at dyrkning med hypoksi influerer den elektrofysiologiske profil på populations niveau fedt stamceller.

Utallige trin er involveret i omdannelsen af udifferentierede stamceller til differentierede, funktionelle celler. Forståelse af molekylære regulatorer, som udgør en del af denne proces er absolut afgørende for udviklingen af den regenerative terapi. Denne afhandling er en lille del af bestræbelsen på at afsløre de molekylære mekanismer bag stamcelledifferentiering, formering og ion kanal funktion.
Selected Abbreviations

ESCs- Embryonic stem cells
ASCs- Adipose-derived stem cells
HIF-α- Hypoxia inducible factor alpha subunit
HRE- Hypoxia responsive element
NICD- Notch intra-cellular domain
mRNA- messenger ribonucleic acid
Q-PCR- Quantitative polymerase chain reaction
TEA - Tetraethylamine chloride
Ir - Rapidly activating current type
Is - Slowly activating current type
Ir+Is - Mixed current type
Kv - Voltage gated K⁺ channel
Kir - Inward rectifying K⁺ channel
HCN- Hyperpolarization-activated cyclic nucleotide gated K⁺ channel
MaxiK- Large conductance Ca²⁺-activated K⁺ channel
α1C- Voltage-gated L-type Ca²⁺ channels
1 Introduction

1.1 Stem cells

The development of an embryo to an individual is driven by a preprogrammed differentiation and proliferation of the cells to form various tissue and organs. However selected group of the cells refrain from participating in this developmental process and remain embedded deep within the organs in what is called the ‘niche’ in a nascent state. These cells have the inherent potential to divide and differentiate in order to cope with the maintenance and repair of the injured, diseased, or dead tissue. These cells are called stem cells. They are unspecialized cells with the unique capacity to self renew and to differentiate into functional, specialized and terminally differentiated cells when induced by specific stimuli.

Stem cells can be broadly classified into: embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst (Thomson et al., 1998) and the adult stem cells, which originate from tissues or organs derived from one of the three germ layers (Fig1.1). For example neural stem cell (Murphy et al., 1997; Galli et al., 2003) hair follicle stem cell (Morris et al., 2004; Ito et al., 2005) limbal stem cell (Schermer et al., 1986; Tseng et al., 1989) are derived from ectoderm, hematopoietic stem cell, bone marrow derived stem cell (Pittenger et al., 1999; Owenet al., 1988), adipose-derived stem cell (Van et al., 1978; Zuk et al., 2001) are derived from mesoderm and lastly pancreatic stem cell (Bonner-Weir & Sharma, 2002) hepatic stem cell (Kuwahara et al., 2008) and gut stem cell (Brittan & Wright, 2002; Brittan, 2004; Potten et al., 1997) are derived from endoderm.
Recently a third category of stem cells, resembling pluripotent ESCs have been derived by inducing terminally differentiated cells to undergo de-differentiation, resulting in the gain of pluripotency. These cells are called induced pluripotent stem cells and have been described for the first time by the Takahashi group (Takahashi et al., 2007).

**Figure 1.1.** Classification of stem cells. Stem cells are categorized as ESCs that are derived from the inner cell mass of the blastocyst stage embryo; adult stem cells that are derives from various tissues from the adult human body and induced pluripotent stem cells that are derived from differentiated human adult cells and are reprogrammed to become pluripotent.

### 1.2 Derivation and characterization

In the following, the derivation and characterization of two types of stem cells namely, ESCs and ASCs will be described as they form the basis of this thesis.
1.2.1 Derivation, culture and characteristics of human ESCs

Human embryonic stem cell was derived for the first time by Thomson et al in 1998. Human ESCs are derived from the inner cell mass of fresh or frozen cleavage stage embryo after informed written consent from donors. The embryos are grown to the blastocyst stage, followed by the removal of the zona pellucida and trophoectoderm and finally the cells from the inner cell mass are isolated by immunosurgery, laser ablation or direct hatching and cultured in special growth media on the top of a layer of mitotically inactivated fibroblast feeder cells (Fig1.2). The inner cell mass derived ESCs can continue to proliferate and replicate themselves indefinitely and also maintain the differentiation potential to form any of the more than 200 cell type of the body.

Addition of basic fibroblast growth factor is vital for the maintenance of undifferentiated state of the human ESCs. However, the presence of non-human components which may pose potential contamination threat in both the substrate on which the cells are anchored as well as the growth medium need to be eliminated in order to make the human ESCs ready for use in cell therapy application. ESCs are typically grown on feeder layers for the maintenance of pluripotency, however in the recent years, several studies have demonstrated the ability of human ESCs to grow in feeder-free conditions, using substrates such as matrigel laminin fibronectin and vitronectin; (Xu et al., 2001; Beattie et al., 2005; Amit et al., 2004; Braam et al., 2008). At the same time, several groups have demonstrated that it is possible to cultivate human ESCs in a defined xeno-free media that is without serum and allows undifferentiated propagation (Ludwig et al., 2006; Chin, et al., 2010; Ellerstrom et al., 2006; Peiffer et al., 2008) making the human ESCs more suitable for future applications.
A summary of the essential features that define ESCs include 1) derivation from preimplantation or periimplantation embryo (ii) ability to self renew that is, prolonged undifferentiated proliferation (iii) pluripotency or potential to form derivatives of all three embryonic germ layers (iv) stable diploid karyotype and continuously express a high level of telomerase activity during the long term propagation in culture (Amit et al., 2000; Thomson et al., 1998).

Characterization includes karyotyping, and verifying the expression of the stage specific antigens SSEA-3 and SSEA-4, the glycoproteins tumor recognition antigen (TRA)-1-60, (TRA)-1-81, and transcription factor Oct4, Nanog, Sox2, among many others (Lysdahl et al., 2006; Cai et al., 2006; Adewumi et al., 2007). However not all tested human ESC cell lines display the same characteristics. This can be due to stage and the quality of the embryo, differences in the method of removal and isolation of the inner cell mass, and varied culture conditions in laboratories. Moreover, each individually derived human ESC line represents an individual human genome and the fundamental characteristics of each line may be determined by its unique genome (Skottman et al., 2006) giving rise to differences in the expression levels of the genes that control pluripotency, self renewal and differentiation (Abeyta et al., 2004).
ZP: Zona Pellucida

TE: Trophoderm

ICM: Inner Cell Mass

Modified from Chen, A. E., Melton, D. A. Derivation of Human ESCs by Immunosurgery J. Vis. Exp. (10), e574, DOI: 10.3791/574 (2007)

**Figure 1.2.** Steps involved in the isolation of human ESC by immnosurgery. Derivation of human ESC line begins with the desolation of the outermost, zona pellucida layer of the blastocyst by the addition of acidic Tyrode’s solution. This is followed by the incubation with rabbit anti-human red blood cell primary antibody and then transfer to guinea pig complement that lyses the trophoderm cells. The residual trophoderm cells are mechanically removed, and the isolated cells from inner cell mass are placed on the feeder layer to enable attachment and outgrowth of the ICM to form compact colonies indicated in the arrows.

1.2.2 Derivation, culture and characteristics of adipose-derived stem cells

Adipose-derived stem cells (ASCs) were isolated for the first time by Van and Roncari in 1978 (Van et al., 1978). ASCs were extracted from the stromal fraction of human omental adipose
tissue by elective abdominal surgery and were successfully differentiated into mature adipocytes in enriched growth medium. The stromal vascular fraction is the lipoaspirate that contains a multitude of cells that include adipocyte precursors, endothelial cells, smooth muscle cells, pericytes, fibroblasts, blood cells and stem cells. The contaminating cells are removed following a number of steps (Fig1.3) that comprise firstly, washing of the lipoaspirate to get rid of blood cells. This is followed by enzymatic dissociation of the adipose tissue with collagenase I or crude mixture of collagenase which releases the ASCs from the extracellular matrix. Thereafter, centrifugation leads to the fractional separation of the ASCs and erythrocytes which sediment to bottom layer of the fractionate. The erythrocytes are removed in the next step by washing with saline solution. The ASCs are seeded in culture flask with growth medium after the additional centrifugation and filtration process. Subsequent medium change after 24 hours remove all the non adherent cells to leave attached adipose-derived stem cell behind. Any other cellular contaminants in the culture gradually die, leaving a pure population of the ASCs in the flask. Isolation of ASC using the above procedure normally results in the recovery of 400,000±200,000 cells per milliliter of the lipoaspirate (Aust et al., 2004; Oedayrajsingh-Varma et al., 2006).
Figure 1.3. Steps involved in the isolation of human ASC from lipoaspirate. The lipoaspirate is washed and then subjected to enzymatic digestion by adding crude collagenase or collagenase. It is followed by centrifugation leading to fractional separation of the stromal vascular fraction. The upper layers are removed and erythrocytes are lysed by saline solution, while the remaining fraction is centrifuged, filtered, and seeded in culture.
The minimal criteria for defining human mesenchymal stem cells is (1) ability to adhere to plastic, (2) differentiation to cells of osteogenic, chondrogenic and adipogenic lineages and (3) expression of CD105 (endoglin), CD 73 (ecto5’ nucleotidase) and CD 90 (Thy-1) antigen marker in more than 95% cells; while the expression of CD34, CD45, CD14, CD19 and HLA class II hematopoietic markers should be less than 2% cells in the total population (Dominici et al., 2006). ASCs being a subclass within the mesenchymal stem cells are also defined according to the above criteria. Additionally, presence of other specific antigen markers such as adhesion molecule like CD 9 (tetraspan) CD29 (integrins), CD49d (integrin alpha subunit), CD54 intracellular adhesion molecule I, receptor molecules like CD44 (hyaluronate), CD71 (transferring), CD144 (cadherin), surface enzymes CD13 (aminopeptidase) extracellular matrix protein collagenase I and III, CD146, α smooth muscle actin, vimentin, complement regulatory protein CD 55 (decaying accelerating factor), and CD59 (complement) protectin are included in the characterization. However variable expression of some markers have been reported, this could be due to subtle differences in the derivation procedure, and it is also possible that the earlier passages may express some hematopoietic markers which are lost with passaging, and fundamental genetic difference within the individual cell lines. Moreover, donor age, site of ASC extraction may also exert some influence over the presence of antigen markers on the surface of the stem cells.

1.3 Role of Oxygen in differentiation, proliferation and ion channel function

In the following the impact of cellular oxygen on the differentiation, proliferation and ion channel functions of stem cells will be described.
1.3.1 The starting point: Hypoxia inducible factor

Oxygen is crucial in cellular respiration of aerobic organisms as it is the ultimate electron acceptor in the respiratory chain that converts biochemical energy by the oxidative metabolism of glucose to adenosine triphosphate (ATP) in the mitochondria. The availability of oxygen has therefore an extensive impact on the cellular mechanisms. When the cellular oxygen demand grows greater than its supply then, it becomes essential for the cells to adapt to the changed micro-environment by lowering their energy consumption via change in the pattern of transcription of several hypoxia responsive genes.

The impact of low oxygen tension on cellular function is brought about by hypoxia inducible factor-1 (HIF-1), which is a master regulator of cellular homeostatic response to hypoxia by activating transcription of genes that are involved in energy metabolism, angiogenesis, apoptosis and other genes that facilitate metabolic adaptation (Semenza, 2003).

HIF-1 is a heterodimer composed of an alpha and a beta subunit (Fig1.4). The beta subunit of HIF-1 is constitutively expressed at all times, whereas the alpha subunit is the oxygen sensitive counterpart. At normal oxygen tension the HIF-1α undergoes hydroxylation of proline (402 and 564) by von Hippel-Lindau protein and asparagine (803) by another hydroxylase called factor inhibiting HIF, (FIH). The hydroxylated HIF-1α is tagged to polyubiquitin, and is subsequently degraded (Semenza, 2004; Taylor, 2008; Patel & Simon, 2008). As oxygen tension decreases to hypoxic levels, HIF-1α is not hydroxylated and thus becomes stabilized. Upon nuclear translocation, HIF-1α dimerizes with the oxygen-independent HIF-1β and binds to hypoxia responsive elements (HRE) within the promoter of the target genes. This is followed by the binding with two co-activators p300 and CREB-binding protein (CBP) to initiate gene
transcription. HIF regulates the expression of at least 180 genes (Semenza, 2000) involved in angiogenesis, glucose metabolism, cell survival, differentiation, proliferation, apoptosis, ion channel function, etc.
Figure 1.4. HIF-1α pathway. In normoxia, hypoxia-inducible factor-1α (HIF-1α) is hydroxylated by pVHL which tags HIF-1α with polyubiquitin and results in subsequent degradation. In hypoxia, proline hydroxylation is inhibited and HIF-1α stabilizes. HIF-1α accumulates and translocates to the nucleus and dimerises with HIF-1β, binds to hypoxia-response elements (HREs) within the promoters of target genes and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity.

The term ‘Hypoxia’ is often used, but has distinct meanings in different contexts. Environmental hypoxia and physiological hypoxia describe two very diverse range of oxygen tension. On one hand, environmental hypoxia means oxygen concentrations below the ambient 21% oxygen found at the sea level, whereas the physiological hypoxia is relative for different tissues and in general refers to a condition when the demand of oxygen is higher than its supply in the cellular
microenvironment. There is a great heterogeneity in the oxygen distribution both between and within the tissues in the body. Various tissues in the body have unequal availability of oxygen in normal conditions. The 21% oxygen concentration inhaled by the lungs is reduced progressively, from about 13% in the alveoli, to as low as 1% in the medulla of kidney (Brahimi-Horn & Pouysségur, 2007). Within the tissue, the heterogeneity in oxygen availability is dependent on the distance of the cells to the closest oxygen supplying capillary and also the cellular metabolic demand. It should be noted that the term hypoxia refers to oxygen tension below 20 % in this study.

1.3.2 Regulation of differentiation by hypoxia and Notch cross talk

Notch signaling is one of the most well conserved juxtacrine signaling pathways among adjacent cells in multi-cellular organisms and is involved in mediating cell fate determination during embryonic development and also stem cell differentiation. The Notch signaling begins when the ligand from the delta-serrate-lag protein family positioned on the extracellular membrane of an adjacent cell comes in contact with the extracellular Notch receptor domain (Fig1.5). Notch receptors are single-pass transmembrane proteins with several tandem epidermal growth factor-like repeats in the extracellular domain, and ankyrin repeats in the intracellular domain (NICD), and also in nuclear localization sequences. Before the Notch complex reaches the cell membrane, the bond between the extracellular and intracellular domain is cleaved in the golgi complex by furin protease (S1). The binding between the receptor and ligand results in the successive cleavages called S2, S3/S4 at different sites mediated first by metaloprotease (S2) followed by γ-secretase mediated cleavage (S3/S4) (Gordon et al., 2008). The final cleavage (S3/S4) leads to the translocation of the Notch intracellular domain (NICD) into the nucleus, where it interacts with the recombination signal binding protein-Jk (RBPJk), along with other co-activators like
p300 or CREB-binding protein resulting in the transcription of Notch downstream target genes like HES and HEY (Tien et al., 2009). The downstream genes regulate the cell’s response to change in the microenvironment either by maintaining cells in undifferentiated state or by initiating lineage commitment, depending on the cell type (Sainson & Harris, 2006; Gustafsson et al., 2005)
Figure 1.5. Notch signaling pathway. Notch is first processed in the golgi complex, where the cleavage by furin protease takes place at the site S1. The processed Notch then binds with the ligand and which leads to a series of cleavage at site S2 by metalloprotease and site S3/S4 by γ secretase. The cleaved intracellular Notch domain translocates to the nucleus and initiates transcriptional activation of Notch downstream genes.

Hypoxia induces Notch signaling which among other things; controls cell fate differentiation in various cell types (Sainson & Harris, 2006; Gustafsson et al., 2005; Prasad et al., 2009; Nekanti et al., 2010). Hypoxia, via a direct interaction of HIF-1α stabilizes the notch NICD, leading to an increase in the transcription of the Notch downstream genes by the recruitment of the HIF-1α to the Notch responsive promoter. A detailed study by Gustafsson and colleagues demonstrated that the differentiation in cell lines like the C2C12, satellite cells, and neural stem cells was abrogated when the cells were exposed to hypoxic conditions. Furthermore this maintenance of
undifferentiated state in the cells was brought about the cross-talk between the HIF-1α and the Notch pathways. A previous study by Ezashi et al, conducted on the H1 embryonic stem cell line has also showed that the differentiation capacity of hESC was widely altered by exposure to 1% and 3% hypoxia (Ezashi et al., 2005). The molecular mechanism leading to this alteration of stem cell differentiation propensity by hypoxia could be explained by the down-regulation of the genes that induce differentiation via the HIF-1α–Notch joint action.

1.3.3 Hypoxia and proliferation

In addition to the effect on Notch signaling, hypoxia has also been shown to influence the proliferation in a wide range of stem cells in a cell specific manner. In some cases, it caused a reduction in propagation rate, for example in human ESCs, 5% hypoxia helped in the maintenance of the pluripotency, however reduced the colony size and population (Prasad et al., 2009) but in most cases for e.g. neural stem cells (Santilli et al., 2010; Gustafsson et al., 2005), human bone marrow mesenchymal stem cell (Grayson et al., 2007), human Wharton’s jelly derived mesenchymal stem cells (Nekanti et al., 2010) low to moderate oxygen tensions increase stem cell proliferation. In previous papers from our laboratory (Rasmussen et al., 2011), we have established that the culture of ASCs in mild hypoxic conditions of 5% oxygen results in increased propagation and the cells are able to maintain their differential potential at the same time. However, apart from the link between hypoxia and Notch signaling, the molecular pathways that are involved in mediating the effects of hypoxia on stem cell proliferation and differentiation are not very well understood.
1.3.4 Ion channels and their regulation by hypoxia

Ion channels are among major effectors of the hypoxia signaling. They are transmembrane proteins found in all cells and are involved in the transportation of selective ions across the cell membrane. Ion channels have been shown to be responsible for the modulation of important cellular functions such as differentiation, cell signaling, cell cycle modulation, proliferation, migration, cell excitability, contractibility, and secretory activity in different types of cells (López-Barneo et al., 2004).

Hypoxia has been shown to regulate the transcriptional and functional activity of specific oxygen-sensitive ion channels in various cell types in a rapid and mostly reversible fashion (Peers, 2002). For example, changes in the mRNA expression level is seen in specific ion channels in PC12 pheochromocytoma cells (Del Toro et al., 2003), pulmonary arterial smooth muscle cells (Shimoda, 2010) and carotid body type 1 cells (J. Patel & Honoré, 2001); while changes in the functional level driven by hypoxia is seen in glumos cells of the carotid body (López-Barneo et al., 2004).

Relationship between the proliferation via cell cycle modulation and a rhythmic hyperpolarization and depolarization has been well presented in a review by Blakiston et al in 2010 in embryonic, somatic and neuroplastic cells (Blackiston et al., 2010). Upregulation of specific ion channel sub-unit genes with increased proliferation in smooth muscle cells has also been observed; while the blockage of the genes lead to inhibition of proliferation and migration in these cells (Cidad et al., 2010; Miguel-Velado et al., 2010). In general, blockade of potassium ion channel, leads to reduction in the proliferation in various cells such as brown fat cells, lymphocytes, hepatocytes, chondrocytes etc. (Pardo, 2004).
However, the effect of hypoxia on the ion channel activity appear to be cell specific, thus inferences from other cell systems cannot be made with regards to effect of hypoxia on ion channel function in stem cells.

On the transcriptional level both ASCs and ESCs express a range of ion channels including several ion channel subunits like Kv2.1 and Kv4.2 found in both cell systems and Kv1.1, Kv1.5, Kv7.3 Maxi K, Kv4.3, and α1 C found in ASCs and α1 A, Kv7.2, Kv9.3, Kv11.1, HCN2 identified in ESCs. Functional expression of delayed rectifier-like currents have been mainly recorded in whole-cell patch-clamp studies in both ASCs (Bai et al., 2007) and ESCs (K. Wang et al., 2005). However, the impact of culturing ASCs or ESCs in mild hypoxia on the ion channel transcription and function has not been studied to date. Moreover, it has not been shown whether the hypoxia-mediated alteration in proliferation is linked to ion channel regulation.

1.4 Basics of electrophysiology

1.4.1 Structure and function of ion channels

Ion channels are selective, very well developed and precision mechanism placed across the cell membrane that allow influx of specific ions into the cells and efflux of other distinct ions out of the cells. The electrochemical gradient across the membrane favors the Na⁺ and Ca²⁺ ions to flow into the cells generating inwards current and the K⁺ ion to flow outwards, generating outwards current (Dublin, 2003).
Activation of the ion channel is brought about by a change in the protein conformation, leading to the opening of the ion channel pore. The channel gating mechanism involves the opening of the channel either by binding of an extracellular or intracellular factor, like hormone or neurotransmitter to the receptor in ligand activated channels or by sensing the change in the membrane potential in the voltage gated channels. The voltage gated ion channels are size and charge selective and also very efficient; $1 \times 10^7$ ions/millisecond pass through activated channels generating electrical signals in the cells.

Cloning of Na$^+$, K$^+$ and Ca$^{2+}$ channels has revealed that they are remarkably similar, suggesting a common ancestry (Zakon, 2012). Previous investigation into the structure has shown that the alpha subunit of the Na$^+$ and Ca$^{2+}$ channels are composed of four homologous domains, comprising six transmembrane segments called S1-S6, whereas the K$^+$ channel has only one domain with six segments and the functional K$^+$ channel is therefore formed by 4 alpha subunits (Fig1.6A and B). The amine and the carboxyl tail of all the domains are positioned in the cytosol. Linker peptide loops join the various segments to form a continuous chain. Some of these linker loops form the receptor site for the binding of ligand. The lumen wall of the channel is formed by the S5 and S6 segments of the four domains. In each domain, these segments are linked by a peptide loop designated as p-loop that dips into the extracellular pore of the ion channel. Each p-loop has negatively charged residues near the mouth/pore of the lumen that attracts the positively charged ions which pass through the channel and is also critical for gating.
Figure 1.6. General structure of ion channels. (A) Side view of tetrameric α subunit of Na⁺ and Ca²⁺ ion channel that consists of four domains. (B) Side view of α subunit of K⁺ ion channel that consists on a single domain. (C) Top view of the pore of the ion channel showing the domain arrangement.

The hydrophobic, positively charged S4 segment is positioned in the center of the domain, surrounded by S1, S2 and S3 from outside and S5 and S6 from inside (Fig1.6C). The S4 and part of the S3 segment forms the ‘voltage sensor’, which senses the change in the membrane potential of the cell. Positively charged arginine residues in the S4 segment move approximately 20 Å outwards in response membrane depolarization, protruding into the external membrane surface. This large displacement, repositions the positive charges directly behind the negative residues of the p-loop, pulling it back resulting in the opening of the lumen (Dublin, 2003; Jiang et al., 2003; Yellen, 2002). All channels open to a diameter just enough to allow the selective hydrated ions to pass through the pore.
During activation, the ions move across the membrane in disrupted bursts rather than in continues flow. Activation leads to the opening and closing to occur in rapid succession and is controlled by the fluttering of the p-loop from the center to the periphery of the pore (due to the attraction of the negatively charged residues on the tip of the p-loop to the positively charged residues of the S4 voltage sensor segment).

Channel activation is followed by inactivation which involves another set of conformational changes in the activated channel, rendering it inactive. This means that the ion channel temporarily becomes insensitive to membrane potential, even though it may be higher than the threshold potential.

1.5 **Principle and technique of whole-cell patch-clamp**

1.5.1 Historical Background

The first real insight into the ion channel apparatus functioning in the excitable cells was given by Hodgkin and Huxley in 1952 (Hodgkin et al., 1952). They described the ionic mechanism leading to the generation and propagation of the action potential, using voltage clamp technique, in the giant squid neuron. The duo received the Noble Prize in Physiology in 1963 for their notable contribution to Science. The next big step in the field of electrophysiology was the invention of patch-clamp method by Bert Sakmann and Erwin Neher in the 1970s (Sakmann et al., 1976). This method revolutionized the cellular physiology study, providing an insight into the passage of ions through the channels generating current in the process. Neher and Sakmann refined the method used by Hodgkin and Huxley enabling them to record the ionic conductance across the membrane in single channels, in frog skeletal muscle cells. They too, received the
Noble Prize for Physiology and Medicine in 1991 for their distinguished work. A cellular recording with higher current resolution was made possible by achieving gigaseal, generated by applying a gentle suction. This improved patch-clamp technique was described by Hamill et al in 1981 (Hamill et al., 1981). The group also described the two new cell-free patch configurations: inside-out and outside in their paper.

1.5.2 Principle of Patch-clamp Recording

Patch-clamp technique is applied for the measurement of ionic currents across the cell membrane via individual or the entire assembly of ion channels (Fig1.7). The patch-clamp technique uses chloride coated silver microelectrode placed in a glass micropipette filled with a solution resembling ionic cellular composition. The micropipette is tightly sealed onto the cell membrane so that the resistance in the cell-pipette junction exceeds 1 giga ohm; this effectively isolates the membrane patch electronically for low noise recordings. Currents flowing through the ion channels in the patch membrane can be recorded by the microelectrode connected to a highly sensitive differential amplifier. A second, ground electrode is immersed in the bath solution and is used to set the electrical potential of the outer side of the cell membrane at 0 mV, while the potential of the inner side of the membrane is controlled by the amplifier connected to the microelectrode.

This technique can be used for measuring either the current across the ion channel(s) in the voltage clamp mode or changes in the membrane potential in the current clamp mode. Voltage clamp recordings rely on the negative feedback mechanism that generates an equal and opposite compensative current to alleviate the difference between the command potential and resting membrane potential. This is the most preferred mode of patch-clamp recording. But when a fixed
amount of current is injected into the cell and the changes in the membrane potential is measured then it is called the current clamp mode. Current clamping does not require a feedback mechanism as it solely records the potential difference (Karmazínová & Lacinová, 2010; Molleman, 2003; Zhao et al., 2008).

**Figure 1.7.** Diagrammatic representation of whole-cell patch-clamp circuit in the voltage clamp mode. The command potential is continuously compared with the measured potential and the difference is compensated by current injection which represents the accurate and opposite current flowing across the membrane and is recorded by the output devise.

1.5.3 Patch-clamp application

The patch-clamp method allows for a real-time, high resolution measurement and recording of ionic currents that flow through multiple or single ion channels across the membrane. Patch-clamping has also enabled the screening and characterization of cells of in particularly for conducting cells. The method is often used for testing the functionality of cells differentiated from stem cells and implanted into animal models. Patch-clamp technology has opened new horizons in the field of biology and medical research. It has become the method of choice for 32
cellular electrophysiological investigations and has enabled researchers to look into the molecular mechanisms of several diseases that are caused due to a dysfunctional ion channels. Characterization of ion channel properties in various cell types and the understanding of the structure-function relationship have also been achieved by this method. And with the emergence of automated patch-clamping, the screening and development of novel drugs has gained momentum. However automated, high throughput patch-clamp systems are very expensive, and on the other hand, the traditional method is time demanding and requires very high skills on the part of the experimenter.

1.5.4 Patch-clamp Configurations

Cell attached mode is the simplest single channel recording configuration, in which the micropipette is placed on the surface of the cell membrane, so that the patch membrane adheres tightly to the pipette (Fig1.8). It is non-invasive, as the cell membrane remains intact; however neither the membrane potential nor the intracellular environment can be controlled using this configuration.

Whole-cell mode is the most preferred, macro-current recording configuration, in which a small suction is applied after the micropipette adheres to the cell membrane, resulting in the formation of gigaseal. Following the formation of the gigaseal, another pulse of suction is applied that causes the patch membrane to rupture; resulting in the establishment of a low resistance electrical and physical continuity between the pipette solution in the micropipette and the cytoplasmic fluid. The pipette solution can easily washout the cytoplasmic fluid as it has a much higher volume compared to the cell volume. It is therefore very important that the composition of the pipette solution is as close to the ionic composition of the cytoplasm as possible to obtain a
physiological recording. The whole-cell configuration also has the advantage that the composition of the fluid media both, inside as well as out the cell can be easily manipulated, giving the possibility of a range of experimental setups and maneuverability.

Inside-out mode is one of the two ‘single channel excised membrane configuration’ described by Hamill et al (Hamill et al., 1981). The micropipette is withdrawn from the cell, after the formation of the gigaseal, resulting in the excision of the patch membrane from the cell, allowing the study the ion channels present on the patch membrane in isolation. Inside-out configuration can be obtained when a vesicle is formed on the mouth of the pipette as it is withdrawn from the cell. The vesicle is then disrupted by briefly taking the pipette out of the bath solution. The membrane facing the intracellular side ends up facing the outside/bath solution. This configuration is used for the study effect of cytoplasmic factors on the ionic current.

Outside-in mode is the second single channel excised configuration in which the pulling away of the pipette after the formation of the gigaseal causes the excised cell membrane to initially break and then fold back and reseal the pipette mouth. This configuration can be used for the study of extracellular factors that affect the ionic currents.

Perforated mode (Horn and Marty 1988) is a variation of the whole patch-clamp cell mode in which the wash-out by the pipette solution due to seal rupture is avoided by perforating the patch membrane so that only small molecules like ions, but not large molecules like various cell organelles can pass though the pores (Horn & Marty, 1988). The pores are formed on the patch
membrane by the addition of membrane perforating agents, for example antibiotics like nystatin, gramicidin D or other substances like hydrophilic saponin β-Escin to the pipette solution.

**Figure 1.8.** Diagrammatic representation of patch-clamp configuration. (A) Intact cell measurement with the micropipette in firm contact with the cell membrane is done in the cell attached mode. (B) Slight suction after the micropipette comes in contact with the cell membrane leads to the formation of the gigaseal. (C) A second pulse of suction disrupts the patch membrane and results in the whole-cell patch-clamp mode. (E) Excision of the membrane patch after the giga seal formation results in the inside-out mode, (D) while excision followed by breaking and resealing of the membrane results in the formation of the outside-out mode. (F) Presence of the membrane perforating agents in the pipette solution leads to the perforated patch-clamp mode.
1.6 Aim of the thesis

The overall goal of the present study is to discern the effects of low oxygen tension on various cellular functions including proliferation, differentiation and ion channel function in the human ESCs and human ASCs. The work encompassing the dissertation is based on the following 3 hypotheses:

Hypothesis I: Culturing human ESCs in 5% oxygen hypoxia can support the maintenance of undifferentiated state. Further, Notch signaling plays an important role in the maintenance of pluripotency.

The study focused on identifying the oxygen tension at which the cells would attain optimal growth with minimal differentiation along with understanding the underlying molecular pathway responsible for the effect. In order to meet the aim, human ESCs were cultures in 5 different oxygen tensions for 4 weeks and the optimal oxygen tension was selected based on the ability of the oxygen tension to preserve pluripotency based on the morphological, indirect immunofluorescence, semi-quantitative and proliferation assays. Additionally the cross talk between HIF-1α- Notch was investigated. This part of the PhD study is presented in Paper I in Chapter 3.
Hypothesis II: Long term culturing of human ESCs in 5% oxygen leads to a continuous and stable maintenance of pluripotency without compromising other cellular properties.

The second study focused at establishing that the human ESCs exposed to long term hypoxia (18 months) maintained features associated with stable undifferentiated propagation and the same time retained the capacity to differentiate into the 3 germ layers. A comparative analysis of cellular properties, such as proliferation, differential potential, chromosomal stability and telomerase activity of human ESCs exposed to long term 20% normoxic and 5% hypoxic oxygen tension was undertaken during investigation. The results from this investigation are presented in Paper II, Chapter 4

Hypothesis III: Stem cells several express ion channel sub-units. Some of them may be involved in the alteration of proliferative properties experienced in cells cultured in low oxygen tension.

The last part of the thesis focused on the ion channel functions of the human ESCs and human ASCs. Ion channels have been demonstrated to affect various cellular functions including cell cycle, differentiation, proliferation, cell motility etc. The study was aimed to investigate the difference in the ion channel expression of the two cell types at normoxic and 5% hypoxic conditions. The regulation of genes expressing ion channel sub-units by oxygen was evaluated. This part of the study is presented in Chapter 5 and Paper III, Chapter 6.
2 Methods and material

A description of all methods and materials used during the studies that form a part of this thesis has been given in individual papers. Detailed information about selected methods is given in this section.

2.1 Semi-quantitative real time PCR and primer designing

Semi-quantitative real time PCR (Q-PCR) was conducted to study the relative expression of various marker genes. All the primers were designed using an open source software, Primer 3, developed by S Rozen and H J Skaletsky in 2000 (http://primer3.wi.mit.edu/). First the mRNA sequence of all the target genes was found in the nucleotide database of NCBI. Same parameters for selecting the primers were used for all the genes. The size of the primers was restricted to maximum 20 bp, and the product size of 50 to 110 was selected. The percentage of CG nucleic acid bases was kept between 45% and 55%. Preferably a maximum self-complementarity of 3 and maximum 3’ complementarity of 1 was used, which was adjusted to 4 and 2 if suitable primers were not found. A CG clamp of 2 and melting temperature between 58°C to 66°C was used.

The specificity of the primers obtained using the fore mentioned parameters was tested using basic local alignment search tool (BLAST) to ensure that the primers did not anneal with any
other sequence. The chances of the primer-dimer formation were checked by using the operon oligo tool. All primers were produced by DNA Technologies, Arhus, Denmark.

The ideal annealing temperature was selected by using a primer optimization procedure in which 8 positive samples with cDNA and 8 negative controls containing no cDNA were used for each primer. The samples were amplified in a two-step PCR protocol with a fixed melting temperature at 95°C and annealing/elongation temperature varying between a gradient from 58°C to 66°C. The PCR run included a melt curve to check that only one product was formed. The temperature at which a single clear peak was obtained from the positive sample and no signal was detected from the negative control was selected.

A 4-fold serially diluted standard curve with a correlation coefficient $R^2 \geq 0.98$, derived by pooling cDNA from all samples and positive controls was used to calculate the relative expression of all the test genes. Each sample was analyzed in duplicates in a single-color real-time PCR detection system (MyIQ, Bio-Rad) using a two-step amplification cycle. The thermocycling protocol consisted of an initial denaturation step of 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C for denaturation and 30 seconds at 60/58 °C for annealing and elongation. In the study with the ESCs, 18S was used as a reference gene; while in the study with adipose-derived stem cells the geometric mean of two reference genes, namely cyclophilin A (PPAI) and tyrosine3/tryptophan 5-monooxygenase activation protein (YWHAZ) was used for normalization.
2.2 Cell culture

For normoxic cultures, cells were grown in standard incubators corresponding to 20% oxygen supplemented with 5% carbon dioxide at 37°C. And for hypoxic cultures, the air was balanced with nitrogen to achieve 1%, 5%, 10%, and 15% oxygen concentrations in separate incubators in a hypoxia glove box workstation (Xvivo System; BioSpherix, NY, USA). The glove box facility with integrated, 5 individually controlled incubators, processing chamber and interconnected microscope chamber with hypoxia control ensured that all monitoring and handling processes were at a constant level of hypoxia without sudden episodes of re-oxygenation.

2.2.1 Culturing human ESCs

Human embryonic stem cell lines CLS1 and CLS2 were propagated by microdissection on a monolayer of γ-irradiated human foreskin fibroblasts in Knockout Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA), supplemented with 20% Knockout Serum Replacement (Invitrogen), 2 mM l glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, 4 ng/mL recombinant human basic fibroblast growth factor (Bio- source Europe, Nivelles, Belgium), 1,000 U/mL penicillin and 1 mg/mL streptomycin. The feeder layer provides certain growth factors that support the undifferentiated state of human ESCs (Villa-Diaz et al., 2009). 2.5 ml of irradiated feeder cell suspension with a concentration of 0.25 million cells/mL was added to 35mm diameter polystyrene culture dishes. The feeder cell suspension was cultured in incubator, with 5% CO₂ at 37°C. The inactivated fibroblast cells attached to the culture dish during overnight incubation. The next day, feeder media and unattached feeder cells were removed by washing with Phosphate Buffered Saline (PBS). 3 ml of hESC growth media was added to the dish to prepare them for human ES cell seeding.
The microdissection method comprised cutting clumps of approximately 0.1 mm square from phenotypically undifferentiated regions of 3 to 4 week old ES cell colonies and transferring the clumps to the polystyrene culture dish with a confluent feeder layer containing 3 ml of hESC growth media. Six such clumps were placed onto each culture dish and carefully transferred to a standard humidified incubator or hypoxia workstation. The entire hESC growth media was changed every alternate day. Prior to media change the media was filtered and kept in the incubator or hypoxia workstation for an hour to bring the temperature to 37°C and equilibrate the oxygen concentration. The colonies were sub-cultured in 4-week intervals.

Two additional cell lines, CSL1-LT and CLS2-LT, that had previously been maintained in such an uninterrupted manner at 5% oxygen for more than 18 months were also included. The dark-field images (Fig2.1) were taken twice a week with a SteREO LumarV12 stereo microscope (Carl Zeiss, Göttingen, Germany) featuring a wide-range zoom capability, and fitted with an AxioCam MRm camera (Zeiss) to record the morphological changes in the ES colonies.

![Figure2.1. Dark-field image of morphological development of a representative colony from CLS-1 cell line cultured at 21% oxygen tension from week 1, 2, 3, and 4.](image)

2.2.2 Culturing human adipose-derived stem cells

The human ASC cultures were established from adipose tissue that was obtained during elective liposuction from three healthy donors and has been described in details previously (Pilgaard et
ASCs were cultured in a growth medium consisting 90% α-MEM (Invitrogen, Denmark), 10% fetal calf serum (Helena Bioscience, UK), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 mg/ml gentamicin (all from Invitrogen). Cells were cultured, in a standard incubator at ambient oxygen (Fig 2.2) or in a hypoxic workstation at 5% oxygen for the hypoxic experiments. The growth media was changed twice a week and the media was kept in the hypoxic chamber for at least 1 hour prior to changing to allow oxygen concentration equilibration. All experiments were conducted on cells at passage 4.

For the electrophysiological measurements, ASCs from passage 2 and 3 were propagated in T-175 flasks to obtain high cell numbers. Subsequently the cell were divided into aliquots of 20,000 and frozen for later use in whole-cell patch-clamp experiment.

![Figure 2.2](image.png)

**Figure 2.2.** Dark-field image of ASCs cultured at 20% oxygen tension.

### 2.3 Electrophysiological measurements

Preparation of the cells for electrophysiological measurements: Four days prior to each session of whole-cell patch-clamping an aliquot of previously frozen ASC was thawed and cultured in
12-well culture plate (Costar, Acton, MA). On the day of the electrophysiological measurement, cells were detached from the surface with a mixture containing 0.01% ethylene diamine tetraacetic acid (EDTA, Titriplex III, Merck Millipore) and 0.125% trypsin (Invitrogen) and suspended in growth medium. Care was taken that the least possible trypsin concentration and incubation time was used in order to minimize cell membrane damage. The cell suspension was transferred to untreated glass cover slips and cells were allowed to settle for 30 to 45 min at room temperature. For the cells cultured in hypoxia, the settling process was performed in the hypoxic workstation. Cover slips were mounted in a custom-built acrylic chamber on an upright microscope (BX51WI, Olympus, Japan) and perfused at 1 ml/min with a Tyrode’s solution at room temperature (Fig 2.3). The Tyrode’s solution contained (in mM) 146 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, and 11 glucose, adjusted to pH=7.4. To assess the effect of the K$^+$ blocker tetraethylammonium (TEA) on whole-cell currents, 20 mM TEA was included in the perfusion solution. For the hypoxic experiments, the perfusion solution was equilibrated with N$_2$, resulting in an oxygen tension of 5±1% in the chamber, as monitored with oxygen meter (OX10, Unisense, Arhus, Denmark).
Whole-cell patch-clamping: Patch micropipettes were fabricated using borosilicate glass (OD 1.5mm, Sutter Instruments Co., Novato, CA) in a programmable Flaming/Brown micropipette puller (P-97, Sutter). The micropipettes were consequently fire polished to smoothen the jagged mouth of the tip. And micropipettes with a tip-resistance between 4 to 5 MΩ when filled with internal solution were used for patching. Internal solution comprised (in mM) 10 NaCl, 1 MgCl₂, 10 HEPES, 5 EGTA, and 120 L-Aspartic acid K⁺ salt, adjusted to pH=7.25.

Semi-attached, rounded cells with intact membrane were selected for patch-clamping. The micropipette was first placed very close to the surface of the cell with the help of a micromanipulator (Fig2.4). A slight and slow negative pressure was put on the pipette resulting in the suction of the membrane into the mouth of the micropipette. This facilitated the formation of a very high seal resistance, in the order of giga ohm between the surface membrane and the
mouth of the micropipette. A second sudden negative pressure was then applied that lead to the puncture of the patch membrane and formation of the whole-cell patch-clamp mode.

![Diagram showing steps in whole-cell patch-clamp mode formation]

**Figure 2.4.** Steps involved in the whole-cell patch-clamp mode formation.

Whole-cell recordings were performed using a patch-clamp amplifier (Axopatch 200B, Axon Instruments/Molecular Devices Corp., Union City, CA). Membrane currents were filtered at 1 kHz using an Axopatch 200B internal low-pass filter and acquired at 5 kHz using a PC equipped with a digitizer board (PCI-MIO-E4, National Instruments, USA) and a data acquisition software (AxographX, Axograph Scientific, New South Wales, Australia). Capacitive and leakage currents were filtered on line by means of the p/n leak subtraction routine in AxographX. Membrane currents were elicited by 300-ms voltage pulses from a holding potential of -60 mV, over the range of -60 mV to 80 mV, in 20 mV increments. To construct the current-voltage curves, the
current values were calculated by averaging the measurements at the end of the plateau phase (150 to 300 ms).

Important considerations and limitations: Whole-cell patch-clamping is a powerful technique that enables the direct recording of multiple or single ion channels. The main drawback of patch-clamping is that it is a low throughput, time consuming and labor intensive technique. However since no other substitutes for the technique is available, it will remain to be an important tool in the field of electrophysiology. The efficiency of this technique can be increased by taking the following points into consideration: Firstly the patch-clamp rig should be placed on top of an anti-vibration pneumatic table to minimize disturbances caused due to even the slightest movement. The membrane of the cells should not be damaged, and the micropipettes should be pulled and polished just before the experiment starts and stored in dust free boxes. Tips that are not smooth or have even a meager coat of dust make the formation of the gigaseal impossible. The microscope with a camera and preferably with a video camera should enable the experimenter to visualize the cells clearly. An inverted microscope should preferably be used in the setup as an upright microscope substantially limits the area between the objective, cells and pipette. In the present case an upright Olympus BW51WI microscope with water immersion objective was used. Although the water immersion objective increased the resolution, the working distance between the objective, micropipette tip and the cell samples was very limited; this meant that the angle of approach of the pipette was always acute. However for achieving a successful whole-cell patch-clamp mode, the tip of the micropipette should have an angle of approach as perpendicular as possible. In addition using an upright microscope also resulted in many pipettes getting damaged during changing from one pipette to another because of limited
space. It is also essential that the micropipette is connected to flexible silicon tubing with connector and syringe for a functional pressure application system. And lastly the setup should be equipped with a perfusion system that allows for the cells to be maintained in a good condition for at least 5 to 6 hours.
3 Amplification of Nanog and ion channel genes in human embryonic stem cell line cultured at 20% and 5% oxygen tensions, with and without Notch suppression

3.1 Introduction

Presence of several ion channels both at mRNA and functional level in undifferentiated human and mouse ESCs was for the first time demonstrated by Wang et al in 2005 (K. Wang et al., 2005). Similar results were obtained by the Jiang group for undifferentiated human iPS cells (P. Jiang et al., 2010).

In general, stem cell differentiation and proliferation are controlled by complex signaling cascades. We have earlier shown that Notch signaling and hypoxic exposure are key factors in the maintenance of pluripotent state in the hESC. And the inhibition of Notch by the addition of γ-secretase inhibitor to the culture media leads to the termination of pluripotent state that had resulted due to the hypoxic exposure in Paper I (Fig5.1) (Prasad et al., 2009)
Figure 5.1. The CLS1 line was treated with γSI during a 3-week normoxic or short term hypoxic culture involving 20% or 5% oxygen concentrations, respectively. The extent of undifferentiated zones was revealed through the expression of Oct4 (green), the areas of differentiation by detection of SSEA1 (red), and nuclei were counterstained with Hoechst 33342 (blue). The images are mosaics, comprised of 30 to up to 72 single fields, at 10-fold magnification. Scale bars indicate 1 mm.

However, the effect of hypoxic exposure on the regulation of ion channel genes and their relation with hESC differentiation has not been studied to date. The aim of the experiment was to study α1 C, HCN2, Kv3.4 and Maxi K gene amplification in human ESCs and to explore the link between maintenance of pluripotency in low oxygen tension and ion channel expression.

3.2 Method and Material

Human embryonic stem cell lines CLS1 and CLS2 were cultured in normoxic and 5% hypoxic conditions for 3 weeks. The γ-secretase inhibitor (Sigma-Aldrich, Brøndby, Denmark) was dissolved in DMSO to achieve 1 μM final concentration and added to the cell culture to inhibit Notch signaling. Total RNA was prepared at the end of 3 weeks of culture with the aid of the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturers’ recommendations and the yield was assessed spectrophotometrically (ND-1000; NanoDrop Technologies, Wilmington, DE, USA). cDNA was made from equal amounts of RNA
input using the iScript cDNA synthesis kit (Bio-Rad), followed by semi-quantitative polymerase chain reaction amplification with gene-specific primers for ion channel genes and Nanog with a protocol already described in Paper III. cDNA from human heart and brain were used as positive control and normalization was carried out with a mean square of GUSB and GAPDH housekeeping genes. The sequence for the primers used is given in table 6.1. GUSB and GAPDH primers were part of a Human Endogenous Control Panel from the TATAA Biocenter (DNA-Technology, Arhus Denmark) commercially available kit

**Table 5.1. List of genes and primer sequence used in this study.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Upstream primer (5'-3’)</th>
<th>Downstream primer (5'-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanog</td>
<td>AGGAAGAGTAGAGGC</td>
<td>CAACTGGCCGAAGAA</td>
</tr>
<tr>
<td>Alpha 1C</td>
<td>TTT CAC CCCATGCC TAC C</td>
<td>CAC TAA AAA GCC CCA CC</td>
</tr>
<tr>
<td>HCN2</td>
<td>GCT TCA CCA AGA TCC</td>
<td>CCA GGT CAT AGG TCA TGT GG</td>
</tr>
<tr>
<td></td>
<td>TCA GC</td>
<td></td>
</tr>
<tr>
<td>Kv3.4</td>
<td>CTG TCA TCG TCA ACA</td>
<td>GTG CTT CTT CCG TTT CTT GG</td>
</tr>
<tr>
<td></td>
<td>ACT TCG</td>
<td></td>
</tr>
<tr>
<td>Maxi K</td>
<td>AAA ACA ACC AGG CTC</td>
<td>AAA CAT CCC CAT AAC CAA CG</td>
</tr>
<tr>
<td></td>
<td>TCA CC</td>
<td></td>
</tr>
</tbody>
</table>
3.3 Results

All the four test groups, namely human ESCs cultured in 5% hypoxia, 20% normoxia, 5% hypoxia with Notch inhibition and 20% normoxia with Notch inhibition were seen to express Nanog, Alpha1c, HCN2, Kv3.4 and Maxi K genes at various levels (Fig5.2).

Nanog was expressed in a statistically significant level in cells cultured in 5% hypoxia compared to the other test group, indicating that it is unregulated in these cells that are essentially undifferentiated.

α1 C was only moderately expressed in all the four test groups, however significant difference in the level of amplification was seen between the cells cultured in 5% hypoxia with and without Notch inhibition and also in the cells cultured in 20%normoxia with and without Notch inhibition. In the case of HCN2 significant up-regulation was seen between the cells in 5% hypoxia and 5% hypoxia with Notch inhibition. No pronounced difference in the level of Kv3.4 expression was observed in any of the test groups. And lastly, a small but statistically significant change in the level of Maxi K gene expression was detected between 20% normoxia and 20% normoxia with Notch suppression.
Figure 5.2. Nanog and ion channel gene expression in response to hypoxia culture with and without Notch inhibition. Expression of Nanog, α1 C, HCN2, KV3.4 and Maxi K genes relative to the GUSB and GAPHD housekeeping genes. Values are represented as the mean and SEM; the asterisks indicate statistical significance, *P<0.05 and **P<0.01.
3.4 Discussion

In line with the Wang and Sartiani group human ESCs cultured in normoxia expressed α1 C gene in the transcriptional level; cells cultured in 5% also showed the same results. Interestingly, induction of Notch inhibition lead to a significant down regulation in both the cases, indicating that maintenance of Notch signaling is one of the requirements for the expression of α1 C in human ESCs.

The HCN family has been shown to control cell cycle proliferation in undifferentiated mouse ES cells via cell cycle modulation in previous investigations (Ng et al., 2010)(Lau et al., 2011). In the human system, HCN2 has been shown to be expressed in the H1 ESC cell line (Sartiani et al., 2007), but however not in human induced pluripotent cell line (P. Jiang et al., 2010). In line with the Sartiani group, we also found that the undifferentiated cells that were obtained from 5% hypoxia express HCN2. And when cells in these colonies differentiated due to Notch inhibition, then the HCN2 was down regulated significantly. The results show that HCN2 is preferably expressed in undifferentiated cell from 5% hypoxia and loss of pluripotent state due to the notch inhibition results in the decreased expression.

Kv3.4 expression has been shown to be regulated by cell proliferation status in human arterial smooth muscles cells. Suppression of this subunit lead to reduction in the proliferation rate; while Kv3.4 knockout resulted in a complete arrest of the cell cycle after the G2/M phase (Miguel-Velado et al., 2010b). On the other hand Kv3.4 has also been found to be regulated by oxygen tension, and was down regulated in acute hypoxic conditions in rabbit carotid body cells (Kääb et
al., 2005). However our experiment showed that there is no difference in the level of Kv3.4 expression between 20% normoxia cells and 5% hypoxia cells, even though the cells cultured in 20% have significant higher proliferation. This indicates that Kv3.4 expression is not controlled either by proliferation or oxygen tension in the human ESCs.

The study by Wang et al on mouse and human ESCs could detect Maxi K expression only in the mouse R1 cell line but not in the human ESCs (Wang et al., 2005). Contrary to their results, we detected the presence of the Maxi k gene in human CLS1 and CLS2 cell lines. We also found that the human ESCs cultured in 20% expressed significantly level of Kv3.4 compared to cells cultured in 20% that were treated with γ-secretase inhibitor to inhibit Notch signaling. This could suggest that in normoxic cultures that expression of Kv3.4 may be linked with the maintenance of Notch in our cells.

In conclusion, the results from the experiment indicate exposure to hypoxia and Notch inhibition could at least in part modulate the expression of some ion channels in the transcriptional level in human ESCs. However further investigation is required to confirm this observation in the functional level. Whole-cell patch-clamping could strengthen the results but as separating our cells into single cell suspension is technically challenging at present, it is difficult to take forward the study.
4 General discussion and conclusion

Stem cells are defined by their ability to self-renew as well as by their ability to differentiate into one or more cell types. In the papers forming the basis for this thesis, we explore to which degree the rate of self-renewal and the rate of differentiation may be influenced by controlling the gaseous oxygen concentration during in vitro culture of embryonic and adipose-derived stem cells.

Oxygen is a one of the critical regulators of the cellular biochemical processes. The intrinsic responses to the lowering of oxygen supply in the micro-environment is regulated by the hypoxia inducible factor HIF-1, which is a master regulator that modulates the expression of genes encoding for enzymes, transporters, growth factors, ion channels, differentiation controlling factors etc. (López-Barneo 2004). In context of stem cells, oxygen has been seen to act as one of the inherent molecular signals that may control the self renewal and differentiation process in the stem cells. In particular, low levels of oxygen regulate the differentiation in many stem cells types (Abdollahi, 2012).

Hypoxia is also a regulator of other important cellular modus operandi including proliferation and ion channel function. Alteration in the proliferative capacity and the ion transport via the porous channels due to the exposure to low oxygen tensions have been observed in several cell types (Shimoda, 2010). However, the effect of hypoxia on the differentiation, cell proliferation or ion channel function in the stem cells relies on the experimental setup in that the effects are very cell type-specific and also largely depends the degree of hypoxia investigated.
In this PhD-study, we first investigated the effect of hypoxia on the differentiation and proliferation of human ESCs cultured in normoxic and hypoxic conditions for short period of 4 weeks during one passage. The understanding gained from the first study was then taken further by studying the properties of human ESCs that were subjected to long term (18 months) hypoxia to evaluate whether low oxygen tension brings about any change in the ESC characteristics like proliferation, expression of differentiation markers, karyotype, telomerase activity, differentiation capacity etc. As ion channels have been implicated in cell proliferation and stem cell differentiation, we then proceeded to investigate first the effect of short term hypoxia on expression of ion channels in human ESC, after which we proceeded to study the effect of hypoxia on both ion channel expression function in human ASCs. The key findings resulting from this work are discussed below.

4.1 Maintenance of undifferentiated state by short term and long term exposure to hypoxia

Two human ESC lines, CLS1 and CLS2 were cultured at 5 different oxygen tensions to determine the oxygen tension at which optimal growth together with minimal differentiation could be achieved (Paper I). We demonstrated that treatment with 5% hypoxia supported the maintenance of undifferentiated human ESC culture as evidenced by a thin uniform monolayer morphology, with negligible differentiated central areas. In comparison, ESC colonies cultured at 20% oxygen were typically larger and had a peri-central thick spontaneously differentiated zone surrounded by a ring of thin, undifferentiated monolayer. However, any conclusions regarding the estimation of number of cells in the colonies cannot be made based on the area of the colonies, because of two reasons (i) the colonies have a three dimensional structure, therefore
cells in the piled up area cannot be accounted for in the measurement of the area (ii) the morphological
of the cells in hypoxia is different to the cells in normoxia, as the cells are smaller, more compact and
less complex in hypoxic conditions (Forsyth et al., 2006). Thus, estimates of the proliferative capacity
of human ESCs, was determined by incorporation of [3H]-thymidine. These experiments showed that
cells in normoxia displayed linear proliferation over the 4 week culture period while the growth rate of
the cells maintained in hypoxic conditions was significantly lower (Paper I). This is in apparent
contradiction to the results published by Forristal et al, where 5% oxygen seems to increase proliferation
in the human ESCs during the first four days of culture, however our results are not directly comparable,
as we investigated the growth rate over a 4-week period.

The observations regarding effects of hypoxia on colony morphology were supported by analysis of
transcription and translation of markers for pluripotent (undifferentiated) and differentiated states.
The hESC colonies cultured in normoxia were sectioned based on their morphology, and the
differentiated and undifferentiated fractions divided prior to mRNA isolation. For the hypoxic colonies
that were entirely undifferentiated, the whole colonies were analyzed. The Q-PCR profiling demonstrated
that the expression of pluripotency marker Oct4, was 4-fold higher in the undifferentiated cells from
21% and 5% in comparison to the differentiated cells. The expression of Nanog followed a similar
pattern; however, Nanog appeared to be present in a higher amount in the colonies grown at 5% than
in the undifferentiated areas from colonies grown at 21% oxygen.
Next, protein level expression of Oct4, a marker of undifferentiated state (Thomson et al., 1998; Niwa et al., 2000) and SSEA1, a marker for the differentiated state (Ezashi et al., 2005; Cai et al., 2006) was analyzed by a two color indirect immunofluorescence. The arrest of spontaneous differentiation by exposure of human ESCs to 5% oxygen tension was also confirmed by this method. The results were in line to those Ezashi and Forsyth. The rate of spontaneous differentiation was evaluated by comparing the Oct4 positive undifferentiated area to the total area of the colonies cultured at various oxygen tensions. The analysis demonstrated that the proportion of undifferentiated area relative to the total colony decreased progressively to approximately 60% when cells were cultured at 10%, 15% and 20% oxygen concentration. The central and peri-central intermediate area of the colonies stained positive for SSEA1 and only the peripheral zone harbored the Oct4 positive cells. Whereas, the hESC colonies cultured in 5% hypoxia almost completely preserved their undifferentiated status with more that 95% staining positive for Oct4 at the end of 4 weeks. The colonies appeared thin and homogenous and consisted essentially solely of Oct4 positive cells.

In conclusion, our study firmly established that culturing human ESCs in mild hypoxia positively contributes to the maintenance of the undifferentiated phenotype and at the same time results in the decrease in the proliferative capacity in 5% hypoxic cultures. This culture conditions thus represents a fine balance between growth and differentiation.

The prevention of spontaneous differentiation by hypoxic culture is in line with findings from other groups (Ezashi et al., 2005; Forsyth et al., 2006; Lin et al., 2006). But the previous studies on the impact of hypoxia on human ESCs properties either was conducted only for 2-4 weeks, or
took place in a manner that allowed for reoxygenation during media change, passaging or morphological observations under microscope. It is well known, that reoxygenation can trigger the generation of the reactive oxygen species that can result in undesirable gene regulation during hypoxic adaptation and also cause chromosomal instability. Therefore a long-term investigation with no incidence of reoxygenation could give a better insight into the effect of low oxygen on the cellular properties of the human ESCs. In our second study, we thus undertook long-term culture of human ESCs, where the cells were passaged, maintained and observed at 5% oxygen for up to 18 months to examine whether the long term continuous exposure resulted in any kind of alteration in the self-renewal, cellular properties or differentiation capacity of the human ESCs (Paper II).

Two of our hESC cell lines, CLS1 and CLS2, were cultured for 25 and 26 passages respectively in continuous hypoxia. Throughout the culture, the colonies displayed a homogenous appearance, consistent with no/low spontaneous differentiation. Semi-quantitative Q-PCR and immunofluorescence confirmed these findings as transcription of Oct4 and Nanog was significantly higher in the undifferentiated parts of the colonies grown at 20% and the colonies at 5%, than in the differentiated part of colonies. Also, indirect immunofluorescence analysis with Oct4 and SSEA1 antibodies, revealed that in the colonies cultured in 5% oxygen an average of 98% of the colony area stained positive positive for Oct4, whereas in the control colonies cultured in 20% normoxia, only 56% of the colony area stained positive for Oct4.

In line with the findings from the first 4-week experiment presented in paper I, even extended culture at 5% oxygen did not restore the proliferative capacity of the hypoxic cells. Throughout
the 18 months, at the end of each 4-week culture period just prior to passaging, the colony area of
the normoxic cultures was on an average 2.6 fold bigger than the hypoxic cultures indicating that
the rate of proliferation in the colonies cultured in normoxia was significantly higher compared to
the hypoxic counterpart. Interestingly, analysis of the telomerase activity indicated that the
undifferentiated cells retained a high telomerase activity associated with high capacity for self-
renewal in spite of the attenuation of proliferation.

Evaluation of the karyotype at the end of the 18 months of hypoxic culture showed no
abnormality in the arrangement, size, shape and banding pattern in the chromosomes of the
human ESCs exposed to long term hypoxia (Paper II), consistent with reports from other groups
that the low oxygen prevents chromosomal abnormalities (Forsyth et al., 2006).

4.2 The effect of hypoxia on maintainance of pluripotentiality is reversible

Interestingly when hESC colonies maintained at 5% oxygen tension were passaged and
transferred back to normoxic conditions, their morphology reversed resembling the colonies
cultured in 20% oxygen tension (Paper I). The ability to reverse phenotype was not lost even
upon extensive culturing at low oxygen. Even after more than 18 months of uninterrupted culture
at hypoxic conditions with no or only minimal spontaneous differentiation, when the colonies
were transferred back to 20% oxygen tension the morphology transformed, resembling a typical
colony cultured in normoxia with central differentiated area and peripheral thin layer of
undifferentiated cells (Paper II). The reversibility of undifferentiated phenotype has also been
documented by Ezashi et al, where colonies cultured in hypoxia for 2 weeks, differentiated upon
return to normoxic conditions. However, contradictory to our result, Forsyth et al., 2006 showed
that the cells that were in 2% hypoxia for 5 passages became sensitized to 21% oxygen and could not survive the switch from hypoxia to normoxia indicating that either, the degree of hypoxia or cell line specific properties influence the sensitivity to hypoxia.

Finally, the capacity of the cells to differentiate into cells from all three germ layers, even after being maintained in an undifferentiated state for 18 months was confirmed (Paper II). Not only the markers of the three germ layers represented by GATA-4 for endoderm, smooth muscle actin for mesoderm and nestin for ectoderm were present in the cells that were obtained from in vitro differentiation; distinct morphological features like epitheloid cells in case of endodermal lineage, fibroblastoid cells in the case of mesodermal lineage and cells with process in case of ectodermal lineage were also detected.

4.3 Molecular mechanisms underlying the maintenance of pluripotency by hypoxia

The molecular mechanism that leads to the maintenance of the pluripotency by hypoxia in human ESCs is still not well established. Previous investigations with H7 human ESC line has shown that Notch activation/signaling is important for the maintenance of undifferentiated state and regulation of the process of differentiation (Walsh & Andrews, 2003). Other studies have demonstrated that the Notch signaling pathway is inactive, but inducible in the human ES cells, and that the cleaved Notch is responsible for the maintenance of differentiating cell types in the human ES cell colonies (Noggle et al., 2006). Interestingly, in a study on various cell types of mouse origin, it was shown that that Notch signaling is required for the promotion of undifferentiated state in stem cells during hypoxic culturing via stabilization of HIF-1α (Gustafsson et al., 2005).
We tested the hypothesis that HIF-1α-Notch cross talk could also be involved in the maintenance of pluripotency during hypoxic culturing in CLS1 and CLS2 hESC line. Our experiments did indeed confirm that hypoxia-mediated up-regulation of Notch1 is involved in maintenance of pluripotency (Paper1). We showed that the level of Notch1 expression in the undifferentiated cells of hypoxic cultured colonies was significantly higher than in the undifferentiated as well as differentiated cells of the normoxic colonies. Furthermore, blocking of Notch signaling in the hypoxic cultures led to differentiation of the colonies. However, when blocking Notch signaling in normoxic cultured colonies, their degree of differentiation was also increased. This indicated that Notch signaling is required for the promotion of the undifferentiated state in both hypoxic and normoxic cultures. Furthermore, in preliminary experiments we found that HIF-1α is only transiently present during the initial days of hypoxia exposure; a finding that was also confirmed by reports published by Forristal et al. (Forristal et al., 2010). As HIF-1 is only present during the first days of hypoxic culture, we could thus conclude that HIF-1 is not directly involved in maintenance of pluripotency of human ESCs during long-term hypoxic culture.

These results suggest that there could be other signaling pathways that may possibly contribute to the maintenance of pluripotency apart from the involvement of the HIF-1α-Notch cross talk. A further investigation on the molecular mechanism controlling this is warranted.

During literature search we found that ion channels are involved not only in the impulse conduction but also in several cellular functions including proliferation and differentiation in several cell types (Deng et al., 2007; Blackiston et al., 2010). We therefore went on to explore the possible link between hypoxia, ion channel expression, and differentiation in human ESCs and
detected a small but significant change in the level of some ion channel gene expression in preliminary experiments. However as it was technically difficult to get human ESCs in single cell suspension for conducting functional studies with whole-cell patch-clamp, so we changed our focus to investigating the impact of hypoxia on membrane currents and ion channel expression of adipose-derived stem cells.

4.4 Culturing human ASCs in mild hypoxia results in alteration of electrophysiological properties of the cells in population level.

Hypoxic culturing of ASCs prior to transplantation for the treatment of ischemic heart and brain diseases is a novel strategy to make the cells more resilient towards the low oxygen environment of the injured tissue. Additionally this has also been shown to improve the survival, migration, proliferation of the transplanted cells (Stubbs et al., 2012; Oh et al., 2010). Several previous studies have documented that both chronic and acute hypoxia exposure can bring about alterations in the transcriptional as well as functional levels in several cell types (Hong et al., 2004; Wang et al., 1997). But to date, it is unknown whether hypoxic culturing affects the electrophysiological compatibility and maturation of transplanted hASCs cells.

The purpose of the third study that forms Paper III was to investigate whether culturing hASCs in mild hypoxia altered the electrophysiological properties. We also investigated whether the ion channel modulation via hypoxia regulation is related to the increased proliferation seen in hASCs cultured in 5% oxygen.
Whole-cell patch-clamp recordings showed that the ASCs elicited potassium ion currents. In our experiments, we show exclusive outward currents, while other groups have also reported, a small fraction of potassium inwards currents and potassium intermediate currents (Bai et al., 2007; Heubach et al., 2004). This could be purely due to the fact these currents are relatively rare and we simply did not come across cells eliciting such currents in the course of our experiment.

The outward potassium currents were categorized according to the ratio of current elicited at 20mV and 60mV as rapidly activating (Ir), slowly activating (Is) and intermediate activating (Ir+Is). The distribution at population level of the three current types was statistically significantly different in cells cultured in normoxia and 5% hypoxia. Additionally the maximum current amplitude at 80mV was also significantly lower in hypoxic cells compared to control.

Previous study from our lab has demonstrated that the hASCs cultured in 5% hypoxia have increase proliferation (Rasmussen et al., 2011). In the present setup, we observed an increase of 44% in the cell proliferation at day 4 for hASCs cultured in 5% hypoxia compared to normoxia. Ion channels and particularly K⁺ voltage gated ion channels have been implicated with control of proliferation via cell cycle modulation in several cell types by other groups (Pardo, 2004; Blackiston et al., 2010). We therefore investigated the role of ion channels in the observed increased proliferation by blocking the potassium currents in cultures with different doses of TEA. We found that the IC50 obtained from both the experimental setups were comparable, and thus concluded that the profile of growth inhibition by TEA was not affected by hypoxia exposure.
Lastly experiments with mRNA amplification demonstrated that transcripts of several ion channel subunits are expressed in hASCs as has also been seen previously in other stem cells (Bai et al., 2007; Heubach et al., 2004; Jiang et al., 2010; Wang et al., 2005). Two other ion channel genes, namely Kv1.1 and interestingly also Kv2.1 which has been shown be sensitive to TEA and oxygen concentration was not expressed in our cells. There was no significant ion channel regulation due to hypoxia culture in the time laps experiments or amplification done only on day 4.

The results indicate that hypoxic preconditioning influences the electrophysiological profile of ASCs on a population level. TEA insensitive potassium ion channels could plausibly play role in hypoxic regulation of increased proliferation in hASCs. The molecular identity of these channels remains to be determined.
5 Strength and limitations of the experiments and future directions.

One of the main strengths of the experiments conducted during the course of study on human ESCs in Paper I and II lied in the fact that we used state of the art BioSpherix hypoxia glove box with integrated microscope chamber for incubating cells at various oxygen tensions which prevented instances of reoxygenation. The human ESCs images for morphological time laps study were taken with variable magnification whereas images for immunofluorescence assay were taken with a minimum of 20 frames that were stitched together to display the entire colony. This gave a very clear understanding of the morphology of the colony and location of differentiated and undifferentiated cells within the colony as they grew from week 1 to 4. Also, it ruled out biases in selecting which frames to present. Also the experiments with reoxygenation of cells cultured for short and long term hypoxia emphasized that culturing human ESCs in 5% hypoxia represents a fine balance between cell proliferation and self renewal and at the same time does not lock the cells in a permanent state of pluripotency. The significance of the findings from the first study were strengthened by investigating the impact of hypoxia on the cellular characteristics of human ESCs cultured in continuous 5% hypoxia for more than 18 months in Paper II.

In the first two studies we used CLS1 and CLS2 human embryonic stem cell lines cultured on top of a layer of human fibroblast feeder cells. Although the feeder cells were of human origin, they invariably introduce several complications in the experimental setup. The colonies had to be scraped off from the feeder layers in the Q-PCR and proliferation assays to avoid inaccuracies due to noise from the feeder cells. This required acute precision and was very time consuming.
Additionally, it was not possible to perform whole-cell patch-clamp experiment on these cells as patch-clamping requires that the cells are in single cell suspension.

In the future it would be interesting to employ a feeder free setup to made the study simpler, that will also enable conducting electrophysiological experiments and on the other hand would have taken away some of the inconsistencies associated with the feeder system. Secondly, it would be worth exploring pluripotency and proliferation of human ESCs in other oxygen tensions like 2%, 3%, 6% and 7% to compare the difference that could arise due to subtle changes in the oxygen microenvironment. Thirdly similar experiments on other hESC line would firmly establish the findings of the study in a global level across several cell lines.

The uniformity of the experiments conducted with hASCs in Paper III was the main strength. All the experiments were conducted with cells from passage 4 of 3 cell cultures, namely ASC12, ASC21, and ASC23, which were grown in normoxic or hypoxic conditions for 4 days. Secondly continuous hypoxic condition during cell culture was maintained by incubating the cells in the BioSpherix hypoxia glove box. And although setting up hypoxic environment in an open system during whole-cell patch-clamping was challenging, it was finally achieved by perfusing tyrode solution bubbled with nitrogen and monitoring the pericellular oxygen with an oxygen-sensor. Another plus point of the investigation was that we used mathematical ratio of current at 20mV and 60mV to classify current types which was relatively straightforward and unbiased. This effectively removed biases and ambiguities in classifying currents based on kinetics, since recordings with atypical characteristics are often encountered.
Limitations in the study include that we could not reach any conclusive result about the identity of the ion channel subunit that could plausibly be involved in the regulation of proliferation via hypoxia signaling because we used only one blocker, tetraethylammonium which is a general blocker of $K^+$ currents and is thought to block a majority of the $K^+$ currents. Secondly investigating the presence of ion channels in a functional level by other methods like western blotting or immunoﬂuorescence could have given additional strength to the results.

In the future, valuable information about the molecular identity of the ion channel involved in modulation of the proliferation via hypoxia could be obtained by using an array of more specific blockers in the proliferation and whole-cell patch-clamp experiments. Additional information about the global ion channel gene expression could be achieved by employing Microarray analysis. This could be followed by immunofluorescent staining or western blotting for specific ion channel subunits that are observed to be regulated by hypoxia in the microarray analysis to confirm the findings on a functional level. And lastly using higher number of cells from more cell cultures in the whole-cell patch-clamp experiment can enable recording rare currents and at the same time give a better representation of the currents elicited by the cells on a population level.
6 References


69


Grayson, W. L., Zhao, F., Bunnell, B., & Ma, T. (2007). Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. Biochemical and biophysical research communications, 358(3), 948–53. doi:10.1016/j.bbrc.2007.05.054


Prasad, S. M., Czepiel, M., Cetinkaya, C., Smigielska, K., Weli, S. C., Lysdahl, H., Gabrielsen, a, et al. (2009). Continuous hypoxic culturing maintains activation of Notch and allows long-term


Villa-Diaz, L. G., Pacut, C., Slawny, N. a, Ding, J., O'Shea, K. S., & Smith, G. D. (2009). Analysis of the factors that limit the ability of feeder cells to maintain the undifferentiated state of


7 Appendix

Paper I: Continuous hypoxic culturing maintains activation of Notch and allows long-term propagation of human ESCs without spontaneous differentiation


S. M. Prasad*, M. Czepiel†, C. Cetinkaya*, K. Smigielska†, S. C. Weli*, H. Lysdahl*, A. Gabrielsen‡, K. Petersen‡, N. Ehlers§, T. Fink*, S. L. Minger¶ and V. Zachar*

* Laboratory for Stem Cell Research, Aalborg University, Aalborg, Denmark,
†Department of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University, Krakow, Poland,
‡Ciconia Aarhus Private Hospital, Hoejbjerg, Denmark,
§Department of Ophthalmology, Aarhus University Hospital, Aarhus, Denmark, and
¶Stem Cell Biology Laboratory, Wolfson Centre for Age-Related Diseases, King’s College London, London, UK

Received 23 January 2008; revision accepted 30 April 2008

84
Abstract

Objective: The maintenance of pluripotency of human embryonic stem cells (hESCs) requires a high efficiency of self-renewal. During in vitro propagation, however, hESCs have a propensity to differentiate spontaneously. In this study, we assessed the nature of hESC responses to hypoxic conditions.

Materials and methods: Human embryonic stem cells were grown in normoxic and hypoxic conditions, and the cells expressing Oct4 and stage-specific embryonic antigen-1 were identified by indirect immunofluorescence. The transcriptional expression of Nanog, Notch1, and Oct4 was determined by a real-time reverse transcription–polymerase chain reaction, and the inhibition of Notch-mediated signalling was achieved with a gamma-secretase inhibitor.

Results: In contrast to culture at 21% oxygen, where the colonies displayed a marked degree of differentiation, we found that during exposure to 5% oxygen, the hESC colonies displayed a homogenous and flat morphology that was consistent with the presence of Oct4-positive phenotype, indicating no spontaneous differentiation. When cultured at 5% oxygen for either 4 weeks or up to 18 months, high levels of Nanog and Notch1 transcriptional expression were detected, albeit the expression was significantly lower during longer exposure. The suppression of differentiation was rapidly reversed on transfer of the hypoxic cultures to normoxic conditions. Looking into the molecular mechanisms of the maintenance of self-renewal at low oxygen
tensions, we found that inhibition of Notch signalling fully abrogated the hypoxic induction of undifferentiated phenotype.

**Conclusion:** Our data, thus, indicate that hypoxic exposure has the capacity to sustain long-term selfrenewal of hESCs and that this effect is mediated through activation of Notch.
Abstract The maintenance of pluripotency of human embryonic stem cells (hESCs) requires a high efficiency of self-renewal. During in vitro propagation, however, spontaneous differentiation occurs frequently, and there is also a risk of chromosomal changes. In this study, we assessed the properties of hESCs after long-term culture at ambient air and 5% oxygen growth conditions. The hESC lines were grown for up to 42 and 18 mo in normoxic and hypoxic conditions, respectively, and their proliferation; expression of Oct4, SSEA1, Nanog, and Notch1; karyotype; telomerase activity; and differentiation potential in vitro were evaluated. In contrast to cultures at 20% oxygen, where the central zones of the colonies underwent spontaneous differentiation, during exposure to 5% oxygen, the hESC colonies maintained a homogeneous and flat morphology that was consistent with the presence of Oct4-positive undifferentiated phenotype. Irrespective of oxygen concentration, the undifferentiated cells expressed high levels of Nanog and Oct4 transcripts, normal karyotype, and high telomerase activity. When assayed for differentiation
potential, they yielded derivatives of all three embryonic germ layers. Our data thus indicate that hypoxic exposure has the capacity to sustain enhanced long-term self-renewal of hESCs. The hESC lines described in the current paper can be obtained for research purposes from the Laboratory for Stem Cell Research, Aalborg University.

**Keywords** Human embryonic stem cells . Hypoxia . Oxygen . Self-renewal . Pluripotency . Long-term culture
ABSTRACT

Background: Hypoxic preconditioning of adipose-derived stem cells (ASCs) enhances several properties, including proliferation and migration, representing a useful strategy to increase the efficiency of cell-based therapies. However, the impact of hypoxia on the electrophysiological properties of ASCs remains largely unknown.

Methods: ASCs were cultured in 5% oxygen tension. After 4 days, whole-cell currents were recorded by patch-clamp. A dose-dependent growth assay was performed using the K+ channel blocker tetraethylamine chloride (TEA). Transcriptional modulation of ion channels during the hypoxic culture was analyzed by RT-PCR.

Results: All analyzed ASCs displayed outward currents, which were classified as rapidly activating (Ir), slowly activating (Is), or mixed (Ir+Is) current type according to their activation kinetics. The distribution of the current types was significantly influenced by hypoxia. While most of the normoxic cells (54%) had a mixed current type, 74% of hypoxic cells displayed the Is current type. Although cell proliferation increased significantly due to hypoxic exposure, the profile of growth inhibition by TEA was not
affected. In addition, none of the analyzed genes encoding for ion channels were found to be significantly affected by hypoxia.

**Conclusion:** The results indicate that hypoxic preconditioning influences the electrophysiological profile of ASCs on a population level, by affecting the activity of ion channels that are relatively insensitive to TEA. Although, the detailed molecular identity of these channels remains to be discovered, this study represents a significant step towards understanding the effect of hypoxia on the electrophysiological properties of ASCs.

**Keywords:** cell proliferation, hypoxia, ion channels, stem cells